THE UNIVERSITY OF ALBERTA

EFFECT OF ELEVATED TEMPERATURE ON GROWTH AND MACROMOLECULAR COMPOSITION OF A PSYCHROPHILE, MICROCOCCUS CRYOPHILUS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

EFFECT OF ELEVATED TEMPERATURE ON
GROWTH AND MACROMOLECULAR COMPOSITION
OF A PSYCHROPHILE, MICROCOCCUS CRYOPHILUS

submitted by Rodney J. H. Gray in partial fulfilment of the requirements for the degree of Master of Science.

To my wife Valerie, and my daughter Kerry Leigh

In full recognition of their patience, understanding and reassurance.

ABSTRACT

A psychrophilic organism, Micrococcus cryophilus, which grew well in glutamate salts medium was studied at temperatures between the optimum and maximum for growth. Transfer experiments were also carried out in which cultures were shifted from optimum to temperatures above the maximum for growth.

Generation time, growth rate, cell size, cell volume and macromolecular composition were studied at the various temperatures.

Results of the compositional studies would indicate that as the temperature is increased above optimum, there is a decline in levels of macromolecules in the culture during the logarithmic phase of growth. When cultures were transferred to temperatures in excess of maximum for growth, and also when the cultures were in the stationary phase of growth, the levels of protein and DNA remained fairly constant, whereas the level of RNA fell drastically. The fall in RNA was most marked at the higher temperatures.

Changes were evident in disc-gel eletrophoretic patterns of cell extracts from cultures grown at 20 or 26^{o}C from those

derived from cultures grown at 27°C, pointing to a qualitative change in the cellular proteins.

It is concluded that the main effect of elevated temperatures on Micrococcus cryophilus is directed towards the RNA component and its associated systems in the cell. It is probable that the primary effect of the elevated temperature is on the synthesis and/or degradation of RNA.

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INTRODUCTION

Gunter (1957) stated, "Temperature is the most important single factor governing the occurrence and behaviour of life". Like other forms of life micro-organisms are susceptible to changes in their physical environment. In spite of their ability to grow under a wide variety of conditions, there are quite stringent restrictions to the variations that a particular species can withstand. Radical departures from a favourable physical environment result in either the death or suspension of growth of the micro-organisms. The elucidation of exactly how temperature is implicated, has presented a subject of research for many in the field of microbiology.

The metabolic activities of an organism are dependent on chemical and physical reactions and the rates of these reactions are influenced by temperature, consequently the pattern of bacterial growth can be profoundly influenced by this condition. In a chemical reaction the addition of energy to the system in the form of heat causes an increase in the velocity of the reaction and further increases in the velocity will occur if more energy is supplied to the system. This state of affairs remains true over an extensive range of temperature

for a chemical reaction, where the reactants and products are not heat labile.

The situation for a chemical reaction is rather analogous to that for an enzymatic reaction, the rate of the latter reaction likewise being increased by an addition of energy to the system in the form of a temperature rise. However, the temperature range over which this will take place is much more limited, since enzymes, being proteins, are subject to heat denaturation. In cellular systems a whole sequence of reactions is involved, all of which have their individual temperature coefficients, therefore a greater degree of complexity is only to be expected. This manifests itself as a deviation from linearity at extremes of temperature. Still, the correspondence between temperature coefficients of chemical and biological reactions is often close. The situation described, can be readily illustrated by reference to the Arrhenius type plot.

Arrhenius developed an equation, which described quite accurately the results he had obtained from an experiment involving the effect of temperature on the rate of hydrolysis of sucrose.

$$V = Ae^{\frac{E}{RT}}$$

V = velocity of the reaction at T^{o} absolute
A = entropy constant.
E = activation energy.
R = Gas constant (1.98646 cals/deg C^{o}/mole)

He later modified the equation, replacing the term E with μ for complicated biological systems.

Taking the natural logarithm of the modified equation one obtains:

$$\ln V = \ln A + \ln e^{-\mu/RT}$$

$$= \ln A + (-\mu/RT)$$

$$\ln \text{rate} = -\mu/RT + C \qquad \text{ie. } \ln A = C$$

From which it is apparent that a plot of ln V against $1/T$ will yield a straight line of gradient $-\mu/R$. $-\mu/R$ = temperature coefficient.

Ingraham (1958) found the temperature coefficient for a psychrophile to be much lower than that of a mesophile, and suggested that this could be due to the presence of enzymes in the former with lower activation energies than in the latter. He further investigated the situation, interested in this particular aspect, but obtained rather negative results. This by no means disproves the presence of such enzymes, since only three were studied and per chance those with the lower activation energies may not have been chosen. In fact, Burton and Morita (1965) have since shown that malate dehydrogenase from the psychrophile Vibrio marinus, has only half the activation energy of the corresponding enzyme from a mesophilic strain of Escherichia coli.

An Arrhenius plot is only linear for medial temperatures and hence investigations outside of this range are of considerable interest. Examination of the structure and composition of the cell, to see whether at extremes of temperature there is any evidence of faulty or inadequate control, seems especially warranted. The industrial significance of heat sterilisation and of freezing and cooling as methods for the preservation of foods, has also contributed to the stimulus for further investigations in this particular direction. The microbiologist is therefore obliged to devote his attention to the behaviour of micro-organisms at temperatures outside their limits for growth, as well as to temperatures within the growth range.

The biological system, as mentioned, is extremely complex, and changes in such things as growth rate of a culture, are the result of the overall effect of temperature on all the chemical reactions involved in the metabolism of the cell. These processes, and the enzymes involved, all have optimum, minimum and maximum temperatures, and it is on the basis of the overall response to temperature that organisms may be separated into three major groups - mesophiles, thermophiles and psychrophiles. Mesophiles and thermophiles are usually characterised on the basis of their growth optima, for mesophiles these fall between

25°C and 40°C, while for thermophiles the range is from 50-70°C. Psychrophiles are a rather ill-defined group of micro-organisms distinguished from the others by their ability to grow at low temperatures, and now generally accepted as "micro-organisms that grow well at 0° within two weeks or have a generation time of less than 48 hours at 0°C" Ingraham and Stokes, (1959). They thus differ from mesophiles in having lower minimum temperatures for growth but their maximum temperatures for growth can vary from around 18°C, as with a strain of Serratia marcescens Kates and Hagen, (1964) to between 40°C and 50°C, which is the range of maximum temperatures for growth of many mesophiles.

What factors are involved in the determination of the maximum growth temperatures for micro-organisms? Why can organisms grow extremely well up to a certain temperature, but if this temperature is exceeded by even as little as 1 or 2 degrees the organism is suddenly unable to exhibit any "true growth"? These are fairly fundamental questions and perhaps, in most cases, almost intuitively the fact is accepted that when a particular temperature is attained a given organism will be unable to grow; but what is the basis for the cessation of growth? In logical sequence to this is the question of whether the low maximum temperatures for growth of organisms like

Micrococcus cryophilus (26°C) and a psychrophilic strain of Serratia marcescens (18°C) have a similar biochemical basis to the higher maximum temperature for growth of other micro-organisms. However, there is a distinct lack of knowledge about the biochemical basis of higher maximum temperatures also, and the amount of information one can therefore glean from this area is rather limited. Nevertheless, some plausible suggestions have been proposed, including the denaturation of enzymes Edwards and Rettger (1937); Sinclair and Grant (1967); Bluhm and Ordal (1969); changes in the properties of membrane lipids Luzzati and Husson (1962); Byrne and Chapman (1964) denaturation and possible degradation of RNA Califano (1952); Strange and Shon (1964); Pace and Campbell (1967) and DNA Marmur and Doty (1959), although in the latter case Marmur has since shown (1961) DNA lability to be unrelated to maximum growth temperature. Selective leakage of cellular components, expecially RNA derivatives, was found to occur at elevated temperatures, accompanied by cell lysis Hagen et al. (1964); Haight and Morita (1966); Wong et al. (1966); Malcolm, (1967); Kenis and Morita (1968); Allwood and Russell (1968). Malcolm has also reported that temperatures of 30°C have no deleterious effect on the activity of all enzymes of the Kreb's cycle in Micrococcus cryophilus. Tai (1967) suggested the most important role in determining the heat sensitivity of

M. cryophilus was played by enzymes associated with RNA synthesis and degradation and in this connection results of Lee, (1968) indicated decreased RNA content in M. cryophilus at 26°C. Byfield et al. (1967) have also described temperature dependent decay of RNA working with a protozan Tetrahymena pyriformis.

In their study of the maximum temperatures for growth of several bacilli, Edwards and Rettger found that the values obtained agreed remarkably well with the temperatures at which the respiratory enzymes (catalase, indophenol oxidase and succinate dehydrogenase) of the bacilli were inactivated. However Rahn and Schroeder (1941) showed that under conditions where 99% of the population was killed by heat only 14% of the peroxidase and 20% of the catalase had been inactivated; they concluded that enzyme coagulation was not the cause of death. Further work was carried out in this field by Evison and Rose (1965) on respiratory enzymes in the psychrophilic strains of three organisms Arthrobacter, Candida and Corynebacterium erythrogenes, which have maximum temperatures ranging from about 22°C (Candida) to 34°C (C. erythrogenes). The respiratory activity particularly of the Candida and the Arthrobacter was adversely affected after transfer of the growing cultures from optimum to a temperature 3-5°C above

maximum. The temperature shift reduced the activities of the T.C.A. cycle enzymes in the organisms, again those of the Candida and the Arthrobacter being most sensitive. Their results, Rose (1965) concludes, thus supported the findings of Edwards and Rettger.

The investigation of Evison and Rose also included examination of the effect of elevated temperature on RNA content; this revealed no evidence for degradation at higher temperatures and they concluded that RNA degradation does not seem to be an important factor in determining the maximum temperature for growth of these organisms.

This view contradicted that of Califano (1952) and Strange and Shon (1964). Califano reported a leakage of RNA from bacteria when, as a buffered suspension, the bacteria were subjected to super maximum temperatures. The temperature at which this process was initiated was dependent on the species but was related to the maximum temperature for growth; an effect which Strange and Shon confirmed in 1964. They found that conditions which accelerated the death rate also increased the rate of degradation of endogenous RNA. In this connection the effect of Mg, which is known to stabilise isolated ribosomes, decreased both rates, whereas EDTA, which accelerates ribosomal breakdown

increased both rates; a strong suggestion of a relationship between rates of death and RNA degradation.

Attention must be drawn to the importance of differentiating between cessation of growth and actual death. These are two conditions whose examination is frequently associated but which are quite obviously two separate effects. This confluence has in fact occurred in the preceeding discussion where in fact Edwards and Rettger were concerned with the effects accompanying maximum growth temperature whilst Rahn and Schoeder were involved in quite a different situation namely temperatures above maximum. Again in this context, Rose has compared work he carried out with Evison to that of Edwards and Rettger's when the treatment Rose and Evison used was in fact lethal; he is for this reason not entirely justified in concluding that the results obtained supported the findings of these earlier workers.

When the growth rate falls to zero at the maximum temperature as shown in the Arrhenius plot, the organisms are not necessarily dead — a statement easily proved by transfer of the organisms back to optimal or sub-optimal temperatures for growth. It has in fact been shown that under conditions of constant temperature in growing cultures death of cells is a rare occurrence; in other words if suitable growth conditions were maintained, as in continuous culture techniques, the vast majority of the cells in the culture would grow indefinitely

and Koch, (1958) suggested the magnitude of death to be considerably less than 1% per hour in a defined medium at 37°C, based on an experiment using Escherichia coli.

Many workers have directed their activities to research on the protein synthesising mechanism in the stressed organism. Purohit and Finnerty (1967) have shown that the ribosomes and protein synthesising enzymes of the psychrophilic bacterium strain 82 are highly labile compared with those of the mesophilic Eschericha coli K-10. Pace and Campbell (1967) substantiate the former result of Purohit and Finnerty in that they observed the thermal stability of ribosomes to be positively correlated with the maximum growth temperatures of the organisms. Similar findings are apparent in work by Haight and Ordal (1969) from studies on Staphylococcus aureus MF 31. Sinclair and Grant (1967) reported temperature sensitive protein synthesising enzymes in the obligately psychrophilic yeast Candida gelida. Studying the same culture Nash et al. (1968) proposed that thermal inactivation of protein synthesis was due to the presence of temperature sensitive amino acyl-tRNA synthetases; out of 13 synthetases examined, 7 proved to be significantly temperature sensitive at 35°C (maximum growth temperature of C. gelida 25°C) the most sensitive being leucyl-tRNA synthetase. Papas and Mehler (1968) found prolyl-tRNA synthetase to be temperature sensitive while Malcolm (1969) reported not only

prolyl but also glutamyl-tRNA synthetase to be temperature sensitive and further suggested these two amino acid activating enzymes to be the molecular determinants of the maximum growth temperature of Micrococcus cryophilus. Both Nash and Malcolm are of the same opinion that possession of thermolabile critical enzymes plays a role in determining the low maximum temperature for growth of psychrophiles but a difference of opinion is evident when Malcolm propounds a structural change in tRNA since Nash tested 10 tRNA species none of which were affected by the treatment used.

The organism used throughout this investigation was originally isolated and described by McLean et al. (1951). It is a large coccus which may occur in chains, clusters and single cells although it was mainly found to exist in the diploid form. Although the organism stained predominantly Gram negative it proved to be Gram variable throughout its growth cycle. Gelatin is not liquefied, indole and H_2S are not formed and nitrates are not reduced. The organism exhibits no haemolysis on blood agar. It is not capable of growth in sodium citrate or in $NH_4H_2PO_4$ as sole sources of carbon and nitrogen respectively. There is no fermentation of xylose, glucose, lactose, sucrose, maltose, cellulose, mannitol, dulcitol or salicin. Urease and catalase are formed but no oxidase. Growth occurs at $-4^{o}C$ and up to

a maximum of 26°C. It is best at pH 7.2 but takes place in the range 5.5 to 9.5. McLean thus classified the organism as a micrococcus and named it Micrococcus cryophilus because of its growth in such a low temperature range.

The taxonomic position of this organism has however been challenged by Mazanec et al. (1966) and Bohacek et al. (1967). Studies by the former group revealed that in morphological structure and cell groupings after division M. cryophilus differed from other Gram positive cocci and resembled the pediococci. The GC content in DNA of M. cryophilus (41.3%) has also shown a noticeable difference from micrococci which have a much higher GC content (65.6 - 75.5%) again being very similar to the pediococci, especially Pediococcus cerevisiae (44.4%).

The inability of M. cryophilus and other psychrophiles to grow at moderate temperatures is truly a fascinating phenomenon. This present investigation was undertaken to elucidate some of the effects of temperature on the growth and macromolecular synthesis of M. cryophilus as the growth temperature was raised from optimum to maximum and above.

MATERIALS AND METHODS

GENERAL

Test Organism and Growth Medium

Micrococcus cryophilus ATCC 15174 (American Type Culture Collection), an obligate psychrophile, was used throughout the course of the experiment.

Stock cultures were maintained at $0^{o}C$ on a minimal medium + 1.5% agar by monthly sub-culture.

The various responses of the organism were studied in a glutamate-salts minimal medium (pH 7.2) Malcolm (1967), in which glutamate provides the sole source of both carbon and nitrogen. It has the following composition:

KH_2PO_4	5.00 gm.	$MnCl_2.4H_2O$	0.01 gm.
Sodium citrate	5.00 gm.	$FeCl_2$	0.001 gm.
NaCl	5.00 gm.	Distilled Water	1 litre
$MgSO_4.7H_2O$	0.08 gm.		

The pH of the medium was adjusted to 7.2 with KOH.

Preparation of Inoculum

At least six transfers, from the mid-logarithmic phase at 20°C, were carried out, before the sub-culture was removed for use in any of this study. This meant that transfers were carried out when the optical density reached 1.5, transfer number one being from the agar slope at 0°C. The carrying out of at least six transfers ensured the use of a culture in which not only was the potential for growth fully expressed, but also one in which the physiological state of the cells would be consistent throughout the series of experiments.

The sub-cultures were maintained in glutamate salts medium in a New Brunswick 'Psycrotherm' incubator shaker (New Brunswick Scientific Co., New Jersey.) in 300 ml baffled flasks shaken at 300 revolutions per minute at 20°C.

Prior to transfer to large incubation flasks, the cells, harvested from the mid-logarithmic phase, were centrifuged at 10,000 x g for 5 minutes at 0°C, resuspended in 0.1M KH_2PO_4/ K_2HPO_4 buffer of pH7, centrifuged and washed a further time in the phosphate buffer and finally centrifuged and resuspended to an optical density of 1 at 600 mμ, a value equivalent to approximately 5×10^8 cells.

Growth Conditions

1200 ml aliquots of glutamate salts medium were dispensed in 2 litre Erlenmeyer flasks fitted with a side arm for inoculation and sampling. A three inch Teflon coated stirring bar was placed in each flask prior to autoclaving at 121°C for 15 minutes.

Since cultures were to be grown at single degree intervals a very precise means of controlling the temperature was necessary. One which would not only permit the adjustment of the temperature to the required value but which would also be capable of maintaining the temperature at such a value within very close limits. To fulfil this requirement an extremely sensitive device was employed, namely a micro-set thermoregulator (Precision Scientific Chicago, Illinois.) with a sensitivity of $\pm$ 0.011°C. The thermoregulator was immersed in a water bath and coupled to a heating and refrigeration system thus maintaining the water in the bath at the desired temperature. The water was circulated from the control bath to a 12" x 12" x 12" perspex (Plexiglas) tank in which the 2 litre Erlenmeyer (culture flask) was immersed to a level such that the circulating water in the tank is above that of the contained medium. In this way the stirred medium was held within very narrow temperature limits.

The stirring bar was activated by placing the water bath plus culture flask on a non heating magnetic stirrer (Bellco Glass Inc. Vineland, New Jersey). In all experiments the stirring bar was rotated at 500 revolutions per minute. A stroboscope (Strobotac 1531-A, General Radio Company, Concord, Massachusetts.) was used to determine the rate of rotation.

The flasks were allowed to equilibrate at the desired temperature, prior to inoculation of a 100 ml aliquot of the washed cell suspension. Samples were removed at precise intervals throughout the growth cycle, acid washed (0.2N $HCLO_4$ at 0-4°C, resuspended in buffer (KH_2PO_4/K_2HPO_4 pH7) and following quick freezing in a CO_2/ acetone bath, held at -20°C until required.

GROWTH

Measurement of Growth

Two parameters were utilised in measuring the growth of *Micrococcus cryophilus*: viable cell count and turbidity.

Viable cell count: dilutions were carried out in 0.1% peptone water at 4°C. Five 0.01 ml samples of a suitable dilution were plated on to the surface of a plate poured at least 24 hours

previous and allowed to dry at 20°C. It was necessary to use surface plates and thus avoid possible heat damage to the cells, by the brief exposure to 45-50°C which occurs when using melted agar for the pour plate techniques Mossel (1964). An incubation time of 96 hours at 20°C was found to be sufficient to facilitate the enumeration of colonies without difficulty. This procedure was used in all the viability studies.

Turbidity: growth of cultures was monitored at precise intervals throughout the growth cycle, by following the change in the absorbance of the culture at 600 mμ in a Beckman DB-G grating spectrophotometer (Beckman Instruments Inc., Scientific Instruments Divisions, Fullerton, California.) using an uninoculated medium blank as a reference. A constant dilution factor of 10 was maintained throughout the range of absorbance measurements thus avoiding errors which occur when various dilution factors are employed.

Measurement of Generation Time and Growth Rate

The generation time g_t is the time required for the culture to double. It is only meaningful during the exponential phase of growth of the culture, when growth rate is constant.

Generation times were determined from semi-logarithmic plots of absorbance at 600 mμ against time, by calculating the time required for the absorbance to double at a given temperature. The cell samples, taken from the incubation flasks at the different stages of growth, were removed via the inoculation port in the flask. At no time was the flask removed from the water bath at its given temperature, nor was the stirrer stopped in order to remove the cell samples, since rapid change in growth rate can occur with little change in the temperature Ingraham (1958) and/or oxygen tension.

The growth rate, k, in hr^{-1}, was calculated from the equation:

$$k = \frac{\ln 2}{\text{generation time}}$$

Measurement of Cell Size and Volume

Photographs were taken of wet mounts under phase contrast of at least four different fields, at a magnification of 400 x, using a Leitz Ortholux microscope fitted with an Orthoplan camera and a Leitz microblitz 300 flash attachment. Contact slides were then made and on projection, the dimensions of the cells were measured with calipers fitted with a vernier scale. Measurements were made of 15-20 cells from each of the various stages sampled, a microscope stage micrometer (Wild Heerbrugg,

Switzerland) with 0.01, 0.10, and 0.50 mm divisions, being used as a reference for final magnification.

From dimensions thus acquired, the cell volumes were calculated, on the approximation that an elipsoid of revolution with those particular dimensions has the same volume as the actual shape of the cell. The equation used was as follows:

$$V = a^2 \times b \times \frac{\Pi}{6}$$

V = volume of the cell
a = long dimension
b = short dimension
Π = 3.142

Total Count

A Total Count was also carried out at the same stages in growth as records of cell size and volume were made. An appropriate dilution of the cell suspension was made in sterile glutamate salts medium and the bacteria enumerated in a Petroff-Hausser counting chamber. (C.A. Hausser & Son, Philadelphia, U.S.A.) Each small square on the counter has the dimensions 0.05 x 0.05 x 0.02 mm thus in 20 of these squares will be the number of million of cells per millilitre. Counts were made of at least 80 of these squares, in determining the number of cells in each sample examined.

ESTIMATION OF MACROMOLECULAR COMPOSITION.

General

Samples for all analyses had previously been centrifuged at 10,000 x g for 5 minutes in a refrigerated centrifuge (Sorvall RC2-B) at 0°C, acid washed in 0.2N $HCLO_4$, (0-4°C) and then twice in 0.1M K_2HPO_4/KH_2PO_4 buffer of pH 7, being finally resuspended to an optical density of approximately 1. By resuspending all samples to approximately the same optical density the composition per millilitre had a much narrower range and was always within the scope of the standard curves prepared.

The Determination of Protein

The protein content of cells were determined colorimetrically after extraction with alkali Lowry et al. (1951) 1.5 ml portions of the buffered suspensions, O.D. approximately 1, were centrifuged for 5 minutes at 10,000 x g in 12 ml tapered tubes. The cells were resuspended to 1.5 ml in IN NaOH, and the tube capped with a marble and placed in a water bath at 37°C for 1 hour. The actual estimation involved 0.5 ml of the alkaline digest + 5 ml of reagent D (prepared by mixing 2% Na_2CO_3, 1% $CuSO_4.5H_2O$ and 2% Sodium tartrate

in the ratio of 100:1:1) which, after 10 minutes at room temperature, was supplemented with 0.5 ml of Folin-Ciocalteau reagent (diluted 1:1 with water). The tubes were left at room temperature for a further 30 minutes when the absorbance at 500mμ was measured. A standard curve was prepared using bovine serum albumin (Calbiochem). Figure 1.

The Determination of Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA)

Introduction

The method used in the determination of the nucleic acids was based essentially on the Schmidt-Thannhauser procedure which provides the best theoretical and practical basis for precise estimation. This method involves the removal of acid soluble nucleotides, sugars etc., digestion in alkali, followed by acidification to effect separation of the DNA from the RNA. The procedure hinges on the fact that whereas RNA is hydrolysed under the digestion conditions employed, the DNA is resistant. The mechanism of the difference in resistance to alkali has been elucidated by Fono (1947) and by Brown and Todd (1952). It is due to the different behaviour towards alkali of the 3,5-

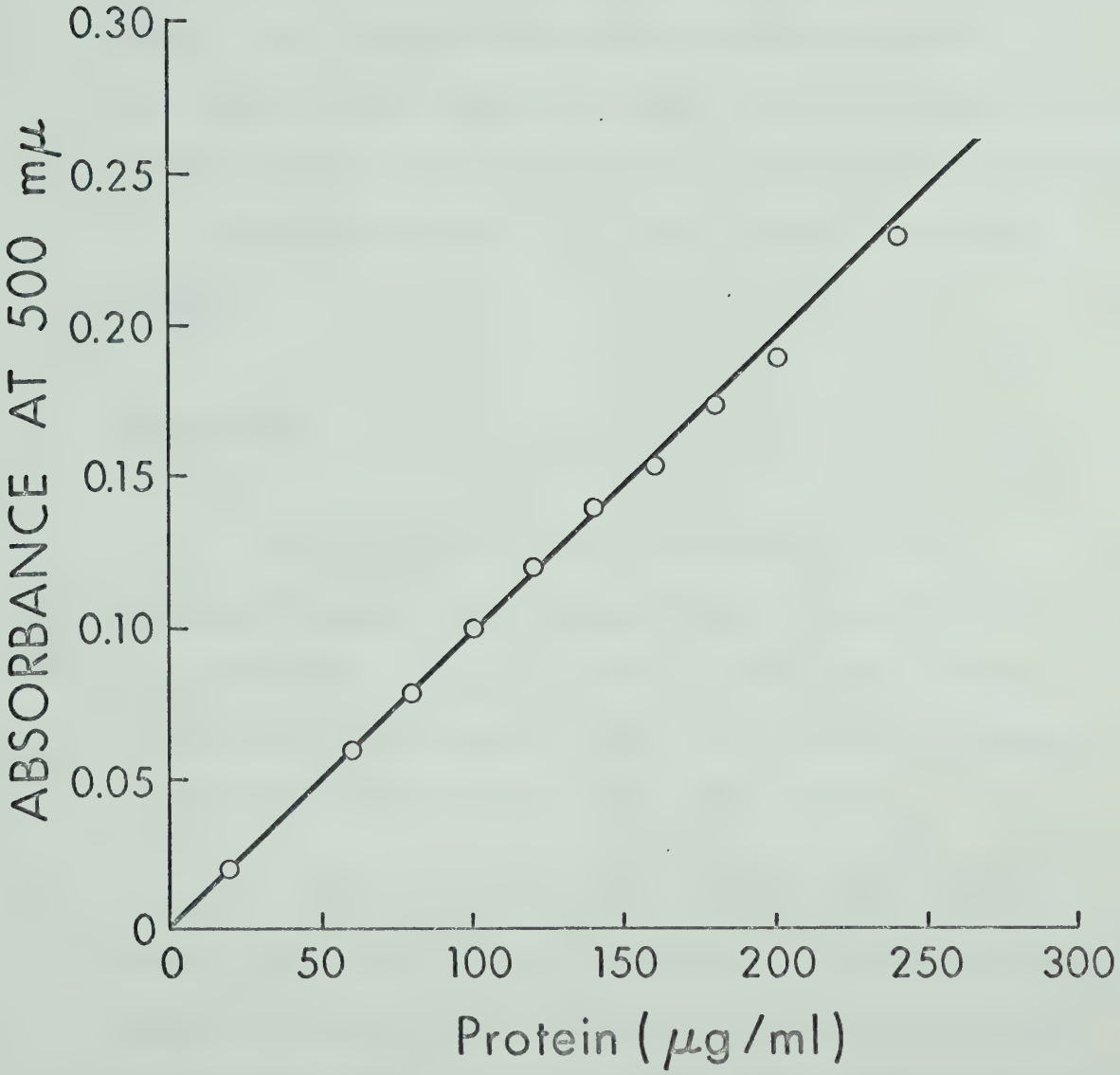


Figure 1.

Standard Curve for the Estimation of Protein.

phosphodiester links between adjacent nucleotides in RNA and DNA. In the case of RNA, on exposure to alkali a cyclic triester is formed with the hydroxyl group on the C_2 of the ribose, which spontaneously hydrolyses to yield 2' or 3' nucleotides. However with DNA, the deoxypentose bearing no hydroxyl group on C_2 in consequence cannot form the essential 2', 3' phospho-triester. It is thus resistant to alkaline digestion.

Determination

The preparation of the cell sample for these determinations took the following form: 5 ml portions of buffered suspensions (O.D. approx. 1) were centrifuged for 5 minutes at 10,000 x g in 12 ml tapered tubes. The cells were resuspended in alkali and incubated in a water bath at $37^{o}C$.

A study was carried out to determine the optimum alkali concentration and the optimum time for digestion of the cells with regard to the subsequent determination of both the RNA and DNA content. Cells from both young and old cultures were tested, in the event different optima for digestion time should present themselves. Values quoted in Table I, are simply the

Effect of Digestion Time in 0.3N KOH on the Determination of RNA and DNA

TABLE I

RNA O.D. 660 mμ

Age of Culture	Digestion Period (hours)					
	1	2	4	6	8	12
12 hours	-	0.186	0.186	0.154	0.129	-
56 hours	-	-	0.310	0.300	0.298	0.299
96 hours	-	0.048	0.075	0.060	0.049	0.049
108 hours	0.025	0.033	0.042	0.030	0.030	0.030

DNA O.D. $(O.D._{595}-O.D._{650})$

Age of Culture	Digestion Period (hours)					
	1	2	4	6	8	12
12 hours	-	0.021	0.023	0.016	0.012	-
56 hours	-	-	0.108	0.091	0.089	0.102
96 hours	-	0.130	0.129	0.063	0.077	0.199
108 hours	0.120	0.130	0.132	0.124	0.104	0.124

colour intensities for the various digestion periods using 0.3N KOH, the optimum alkali concentration. From this data, a time of four hours for the digestion period was judged most suitable, and the procedure used was as follows.

The cells were resuspended in 2 ml 0.3N KOH, and incubated in capped tubes in a water bath at 37°C for four hours. After cooling to 0°C, to facilitate maximum precipitation on addition of acid, 1.25 ml of 1.2N $HClO_4$ was added, and the preparations allowed to stand for a further 10 minutes in ice. The samples were centrifuged and the resulting supernatant transferred to a graduated centrifuge tube for RNA estimation. The precipitate was washed with 1.25 ml of 0.2N $HClO_4$, centrifuged and the supernatant added to the RNA sample. The RNA sample was then made up to a final volume of 5 ml; 2 ml aliquots of this being used in the colorimetric estimation.

Difficulty was experienced, in redissolving the precipitate resulting from the acidification of the alkaline digest with 1.2N $HClO_4$. The recommendation of the original procedure to use 0.3N KOH for this purpose proved to be

unsatisfactory; even when shaken with the alkali for various lengths of time up to 4 hours the RNA remained almost undetectable. 0.5N $HClO_4$ was tried, and proving to be somewhat more promising a time/temperature study was carried out. The precipitates from one original sample, treated in an identical manner, were heated with 0.5N $HClO_4$ at 70, 80 and 90°C for periods of 10 and 15 minutes at each temperature. The results of this study showed 80°C for 10 minutes to be the optimum conditions for extraction of the DNA. Evidence of deoxyribose degradation at 90°C and possibly even at 80°C was noted, with incomplete extraction taking place at 70°C, thus emphasising the importance of careful examination to indicate optimum conditions. A further study using concentrations of $HClO_4$ from 0.01-0.80N indicated 0.50N to be the optimum concentration. Thus the following procedure was arrived at.

The precipitate was dissolved in 2.5 ml 0.5N $HClO_4$ at 80°C for 10 minutes. This preparation was then centrifuged, and 1 ml aliquots of the supernatant used for the DNA estimation, by a slight modification of the Burton (1956) procedure.

The RNA content was determined by the orcinol method Schneider (1957) using the 2 ml aliquots from above + 2 ml 0.2N $HClO_4$ and adding to this 4 ml $FeCl_3$/HCl reagent (0.1%$FeCl_3.6H_2O$

in conc. HCl). This was mixed and 0.4 ml orcinol reagent added (10% orcinol in 95% ethanol). The mixture was then heated in capped test-tubes for 30 minutes in a boiling water bath, and the intensity of the green colour read at 660mμ in a DB-G spectrophotometer. A standard curve was prepared using D-ribose (Calbiochem). Figure 2.

The 1 ml aliquots for the DNA determination were mixed with 2 ml diphenylamine reagent, (1 g diphenylamine in 100 ml of glacial acetic acid plus 2.75 ml H_2SO_4 supplemented with 0.5 ml of aqueous acetaldehyde solution (16 mg/ml) immediately before use) Burton (1956). The tubes again capped with marbles were kept at 30°C for 16 hours, after which the absorbance was measured at 595 mμ and 650 mμ Dische (1955). The difference is then related to deoxyribose content using a standard curve prepared with 2-deoxy D-ribose (Calbiochem) Figure 3. The use of this two wavelength system results in the elimination of the effects due to interfering compounds, which could not be accounted for if measurements were carried out at only one wavelength.

QUALITATIVE STUDY OF SOLUBLE PROTEIN

Preparation of Cell-free Extracts

At each given temperature the cells were grown for two

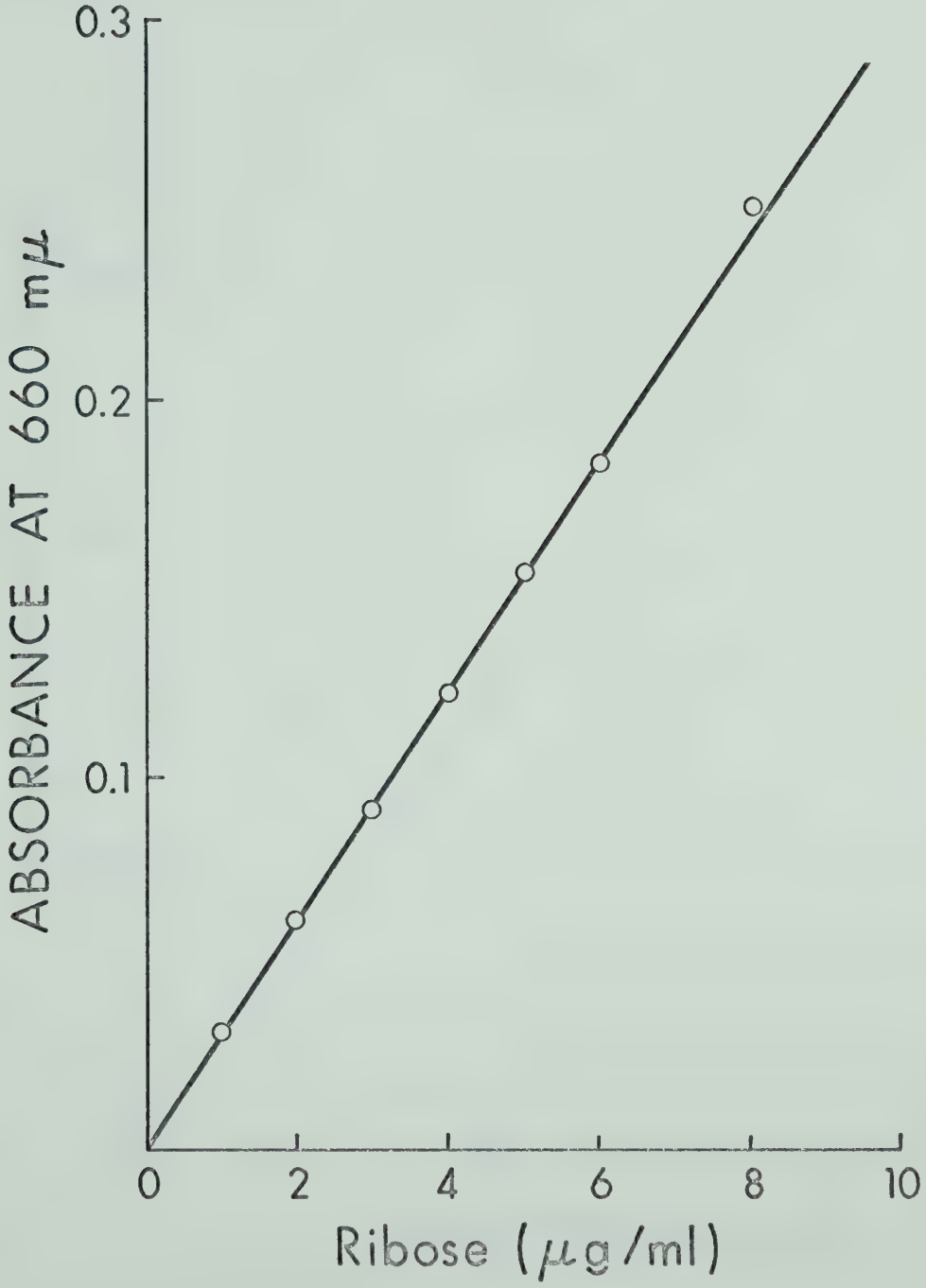


Figure 2.

Standard Curve for the Estimation of RNA.

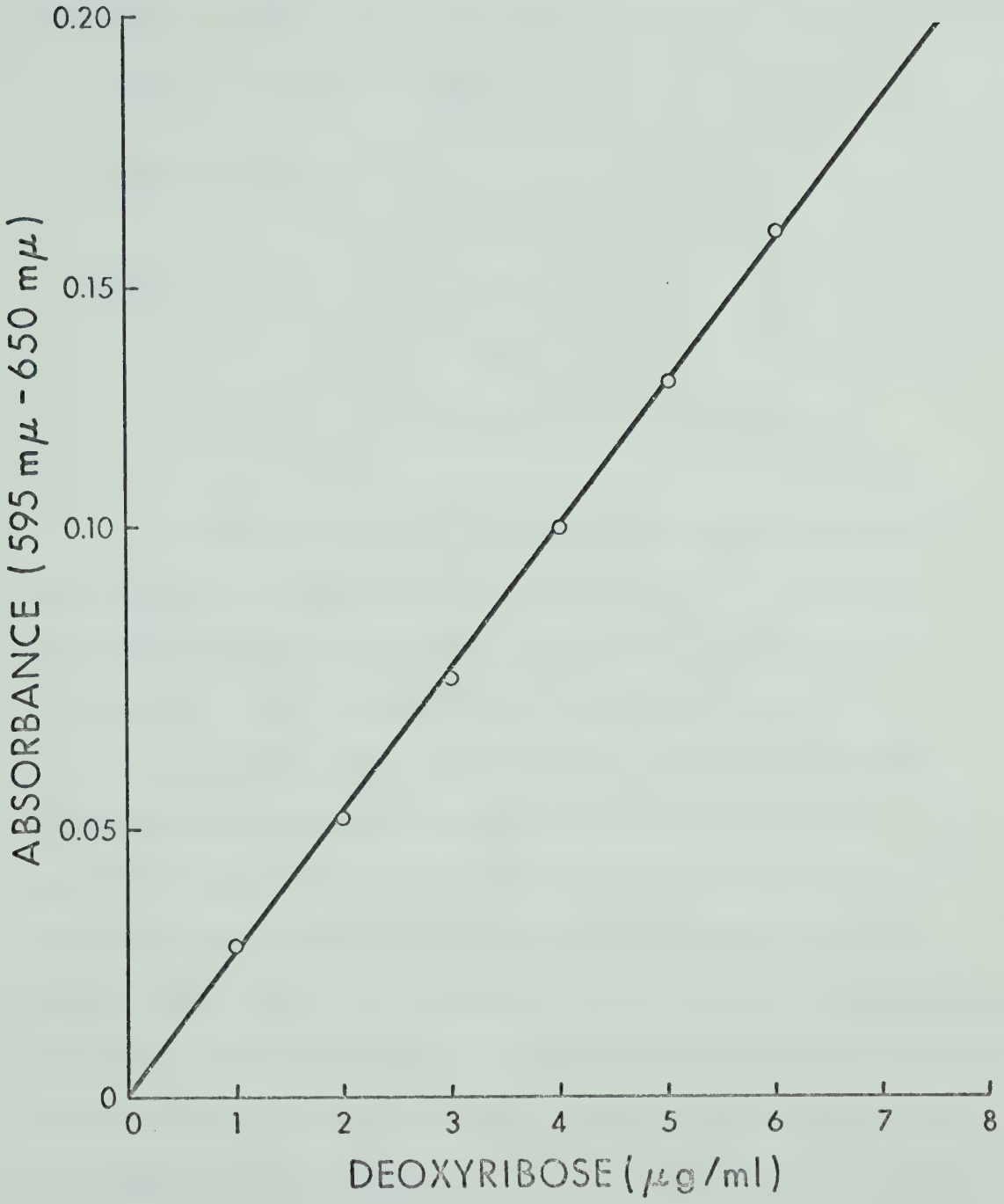


Figure 3.

Standard Curve for the Estimation of DNA.

different periods of time, providing in each case a mid-logarithmic culture and a stationary culture.

Stage of Culture	Temperature of Incubation	Growth Period (hours)
mid-logarithmic	20°C	16
	26°C	20
early stationary	20°C	44
	26°C	54

A culture was also grown at 27°C for 22 hours.

At the appropriate time the cells were harvested and centrifuged at 10,000 x g for 5 minutes at 0-4°C. The resulting pellet of cells was combined and washed with Tris buffer pH 7.8 (0.1M Tris, 10 mM Mg^{++} and 50 mM KCl). The cells, in a thick slurry form, were then added to a French pressure cell which had been pre-cooled to 0-4°C. A pressure of 15,000 pounds per square inch was applied for a period of at least 5 minutes using a standard laboratory press (Carver Laboratory press). When this time had elapsed, the cells were ejected through the small orfice as smoothly as possible by careful regulation of the release valve and concurrent maintenance of the pressure. The highly viscous suspension resulting from the cell breakage

was kept in ice, and the procedure was repeated twice to ensure thorough breakage. Cell-free-extracts were obtained by centrifuging the suspension of disrupted cells at 30,000 x g for 30 minutes at $0^{o}C$. The supernatant was removed and further centrifuged at 105,000 x g for 2 hours in an ultracentrifuge (Beckman Ultracentrifuge Model L2 65B) to pellet out the ribosomes, the supernatant containing the soluble protein. The protein contents of the extracts were determined by the method of Lowry et al. (1951).

Disc Electrophoresis

Introduction

Ionic mixtures can be effectively separated by zone electrophoresis, a method depending for its function on the differential electrophoretic mobilities of the constituent ions. However, in cases where differences among the free mobilities are small, satisfactory separation may not be achieved. A gel matrix is a latticed structure with pores of molecular dimension that can impose an appreciable frictional resistance to the passage of ions, provided the size of the pores approaches the dimensions of the migrating ions. Such is the case with polyacrylamide gels, where the average pore size approaches the range

of dimension of proteins. Differential retardation, in proportion to their dimensions, is thus effected on the various protein fractions; separation now being the result of both charge and dimensional differences. Polyacrylamide gels are synthetic polymers formed from low molecular weight chemicals obtainable in high purity. They are relatively inert chemically; mechanically strong over a wide range of pore sizes; and in the visible wavelengths are transparent.

The technique of disc electrophoresis, a specific form of polyacrylamide gel electrophoresis, is so named because the bands or zones stack up as a series of concentrated discs at the commencement of the procedure and not because of any discontinuity of buffer systems which may be used in the technique, nor because the proteins are located as a series of discs at the end of the experiment. This stacking occurs in the first of two gels, the ions migrating solely on the basis of electrophoretic mobility. Thus faster ions such as proteins will be ahead of slower ions such as weak acids or bases, the sample being concentrated into a very narrow zone enhancing subsequent resolution.

At this point the sample ions are arranged to migrate into a second gel, where a different set of conditions of pH

and pore size exist, so that the mobility of the ions of the weak acid or base now exceeds that of the fastest protein. These ions continuously overtake and pass through the sample species, establishing a comparitively uniform voltage gradient in which electrophoretic separation of the sample occurs.

Apparatus

The entire electrophoretic study was carried out using a Polyanalyst (Buchler Instruments Inc., Fort Lee, New Jersey). The electrophoresis was performed in twelve glass tubes 75 mm long x 5 mm I.D. x 8mm O.D., firmly screwed into rubber grommet-lined holes of the tube holder assembly which forms the floor of the upper buffer chamber. All tubes are equidistant from one another and from the electrodes, ensuring the voltage drop between electrodes and each of the tubes is the same.

Reagents

The stock solutions presented in Table II were stored in brown bottles under refrigeration; they are stable for extended periods of time under these conditions. The working

solutions shown in Table III were prepared on the day of use. The buffer solution shown in Table IV was used to fill the reservoirs.

TABLE II

Stock Solutions Used for Gel Electrophoresis

Solution A		Solution B	
1N HCl	48 ml	1N HCl	48 ml
TRIS	36.6 gm	TRIS*	5.98 gm
TEMED*	0.23 ml	TEMED*	0.46 ml
8M UREA to 100 ml (pH 8.9)		8M UREA to 100 ml (pH 6.7)	
Solution C		**Solution D**	
Acrylamide**	28 gm	Acrylamide*	10 gm
BIS*	0.735 gm	BIS*	2.5 gm
8M UREA to 100 ml		8M UREA to 100 ml	
Solution E		**Solution F**	
Riboflavin*	4 mg	Sucrose	40 gm
8M UREA to 100 ml		8M UREA to 100 ml	

*TRIS = tris (hydroxymethyl) amino methane (Fisher Scientific Co.)

*TEMED = N,N,N^1,N^1 - tetramethylethylenediamine (Eastman Org. Chem. Co.)

**Acrylamide = $CH_2CHCONH_2$ (Eastman Org. Chem Co.)

*BIS = N,N^1- Methylenebisacrylamide (Eastman Org. Chem. Co.)

*Riboflavin - (acting as a catalyst with solution B) (Eastman Org. Chem. Co.).

TABLE III

Working Solutions used for Gel Electrophoresis

A. Large pore gel

B	1.0 ml	
D	2.0 ml	mixed gently = large pore gel solution pH 6.7
E	2.0 ml	
F	4.0 ml	

B. Small pore gel

Solution 1.

A	10 ml
C	20 ml
H_2O	10 ml

Solution 2.

Ammonium persulphate 0.14 gm

H_2O to 100 ml

(acting as a catalyst with Solution 1).

Solutions 1 and 2 were mixed in the ratio 1:1 just prior to the transfer of the gel solution to the tubes. Mixing was again carried out gently to avoid the incorporation of air bubbles. The resultant mixture is the small pore gel solution pH 8.9

TABLE IV

Buffer solution for reservoirs in gel electrophoresis

TRIS*	6.0 gm	= pH 8.3
Glycine	28.8 gm	
Water to 1000 ml		

1/10 of the strength of this stock buffer is used to fill the reservoirs.

---

*TRIS = tris (hyrdoxymethyl) amino methane (Fisher Scientific Co.)

The position of the protein bands was demonstrated using an Amido Schwartz fixative stain in acetic acid (Amido Schwartz, 0.1 gm in 7% acetic acid 100 ml). A tracking dye bromophenol blue (bromophenol blue 0.005 gm in H_2O 100 ml) revealed the progress of migration in the gel tubes, and is used as the zero line in the densitometer traces. Destaining and staining of the gels were carried out using 7% and 3% acetic acid respectively.

Procedure

Stock solutions were allowed to warm to room temperature ($20^{o}C$) before use. The detergent (Sparkleen) and Photo-Flo (Eastman Kodak Company, N.Y.) treated gel tubes were set up in the polymerisation rack and the lower ends plugged with the special rubber corks.

0.2 ml of 40% sucrose was added to each tube, prior to the addition of the large pore gel solution, which was prepared at this time. Using a special syringe 0.2 ml of the latter solution was gently transferred to each tube, care being taken not to cause mixing with the 40% sucrose. The large pore gel was layered with about 0.5 ml of H_2O by allowing it to run slowly down the wall of the tube; the water settling on top of the denser gel solution prevented the formation of a meniscus

on the gel. The gels were then polymerised with a daylight fluorescent light at a distance of 5 cms, exposure being for 30 minutes. During polymerisation, the small pore gel was prepared according to the procedure outlined in Table III. The water layer was removed from the polymerised gels by inverting the polymerisation rack and rinsing the tubes twice with the small pore gel solution. The tubes were then filled completely with the latter solution, an extra drop being added to each tube to give a high meniscus, thus allowing for subsequent shrinkage in polymerisation. The tubes were placed in the dark and a 30 minute period allotted for gel formation. At the conclusion of this time the rubber corks were gently removed and the first layer (40% sucrose solution) discarded. The tubes were then placed in the corks of the upper reservoir.

The lower reservoir was filled with a chilled 1:9 dilution of the stock buffer solution (Table IV) and the electrode connected to the anode. The same buffer solution containing about 0.5 ml/tube tracking dye was then added to the upper reservoir. The sample mixed in the ratio 1:2 with 40% sucrose (80 µg protein total) was applied through the buffer using a long needled syringe and the cathode finally connected to the power supply.

The power supply was adjusted to and maintained at 2 milliamps/tube until the tracking dye reached the small pore gel (20 minutes) when the current was increased to 4 milliamps/tube. Electrophoresis lasted a total of 45 minutes by which time the tracking dye reached an average position 1.5 cm from the end of the tube. The runs were carried out in a cold room at $4^{o}C$; the temperature of the buffer solution rose no more than 4 centrigrade degrees throughout the experiment. In general the procedure is similar to that of other workers Davis (1965).

At the completion of electrophoresis the gels were removed from the tubes by gently "rimming" them under running water, with an hypodermic needle. The gels were immediately immersed in the 0.1% amido schwartz fixative solution for 60 minutes. Subsequent to this the gels were washed for 30 minutes in 7% acetic acid to remove any excess stain. Electrophoretic destaining was then performed, the gels contained in tubes with slightly constricted ends of I.D. 6 mm. A current of 12 milliamps/tube was applied for 60 minutes, facilitating the removal of unbound dye from the gel.

The destained gels were stored in stoppered glass tubes 10 x 75 mm filled with 3% acetic acid. Band positions were

scanned in a Chromoscan Universal Recording and Integrating Densitometer (Joyce, Loebl and Co. Ltd., Heston, Middlesex, London) and also recorded photographically. A diagrammatic presentation was made of each gel making best use of the two methods employed as well as visual examination.

The majority of the preliminary electrophoretic work was carried out using the above method. However the results obtained left much to be desired, problems being experienced with band distortion and poor resolution. In light of this, certain modifications to the procedure were subsequently carried out.

The most important modification was to rely solely on the use of the small pore gel for separation and resolution of the various protein fractions. This, combined with a constant current supply of 3 milliamps/tube throughout the whole experiment eliminated the problem of band distortion. Electrophoretic destaining, used occasionally in the first method, had proved to be very unsatisfactory and was abandoned completely. In place, the gels were destained by immersion in 7% acetic acid for 18 hours using a rotary shaker to provide gentle agitation throughout the destaining period.

RESULTS

GROWTH

The growth of M. cryophilus incubated at various temperatures from 18-30°C are illustrated in Figure 4; the data are based on duplicate determinations on individual samples from at least two complete runs. Since the organisms were sub-cultured at least six times and transferred from the same medium while in the mid-logarithmic phase of growth no lag phase was observed, as measured by absorbance at 600 mμ. The behaviour of the culture for temperatures 18 -26°C was very similar, the growth rate progressively decreasing as the temperature was increased above 20°C, the optimum for the organism. A most conspicuous change however was evident, between the 26°C curve and that for 27°C, delineating the maximum growth temperature of the organism. Since the method of temperature control was very precise the maximum growth temperature for M. cryophilus could be determined more accurately than on previous studies carried out on this organism. It can be seen that M. cryophilus ceased to grow exponentially above 26°C, although at 27°C the cells were able to multiply approximately three times before succumbing to the effect of the higher temperature. If, for a definition of growth we require the organism to exhibit an

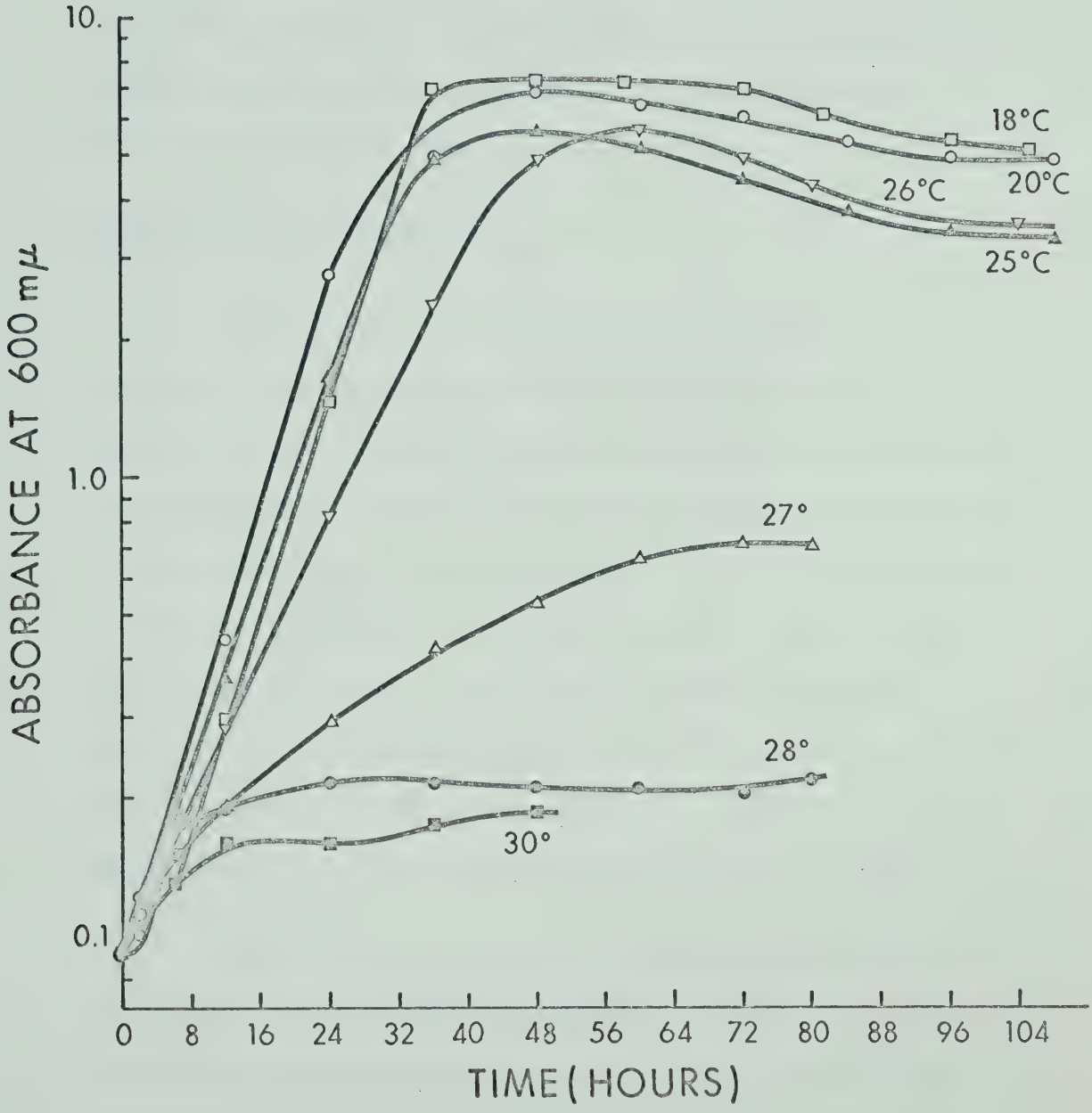


Figure 4.

Effect of Temperature on the Growth of M. cryophilus.

exponential phase then the maximum growth temperature for this organism is 26°C. At 28°C and 30°C the cells were able to divide once before entering the stationary phase, demonstrating the deleterious effect of the unfavourable environment on the metabolism of the cells.

GROWTH RATE AND GENERATION TIME

The changes in growth rate and generation time with growth temperature in glutamate salts medium are presented in Table V; the data are calculated from observations of at least 2 complete runs at the various temperatures. The generation time of M. cryophilus in the exponential phase at 20°C was 275 minutes, this time increased progressively with rising temperature until at 26°C it was 460 minutes. Since there was no exponential phase at 27°C quotation of a value for generation time would be rather meaningless since it only applies to an infinitesimally small period of time.

Values for the growth rate and generation time of M19 (a mesophilic mutant of M. cryophilus isolated by Tai, 1967) in Trypticase Soy Broth are also presented, for use as a basis for comparison.

The results for generation time are also presented graphically in Figure 5.

TABLE V

Generation Time (g_t) and Growth Rate (k) of M. cryophilus (ATCC 15174) and a mesophilic mutant M19 in glutamate salts medium.

Incubation Temp. (°C)	g_t (mins.)		k (hr^{-1})	
	ATCC 15174	M19†	ATCC 15174	M19†
17	-	300	-	0.139
18	314	-	0.132	-
20	274	240	0.152	0.173
25	343	-	0.121	-
26	460	-	0.090	-
27	-	120	-	0.346
30	*	100	*	0.416
32	*	105	*	0.396
34	*	140	*	0.297
37	*	290	*	0.143
40	*	∞	*	∞

* No growth observed

† From Tai (1967)

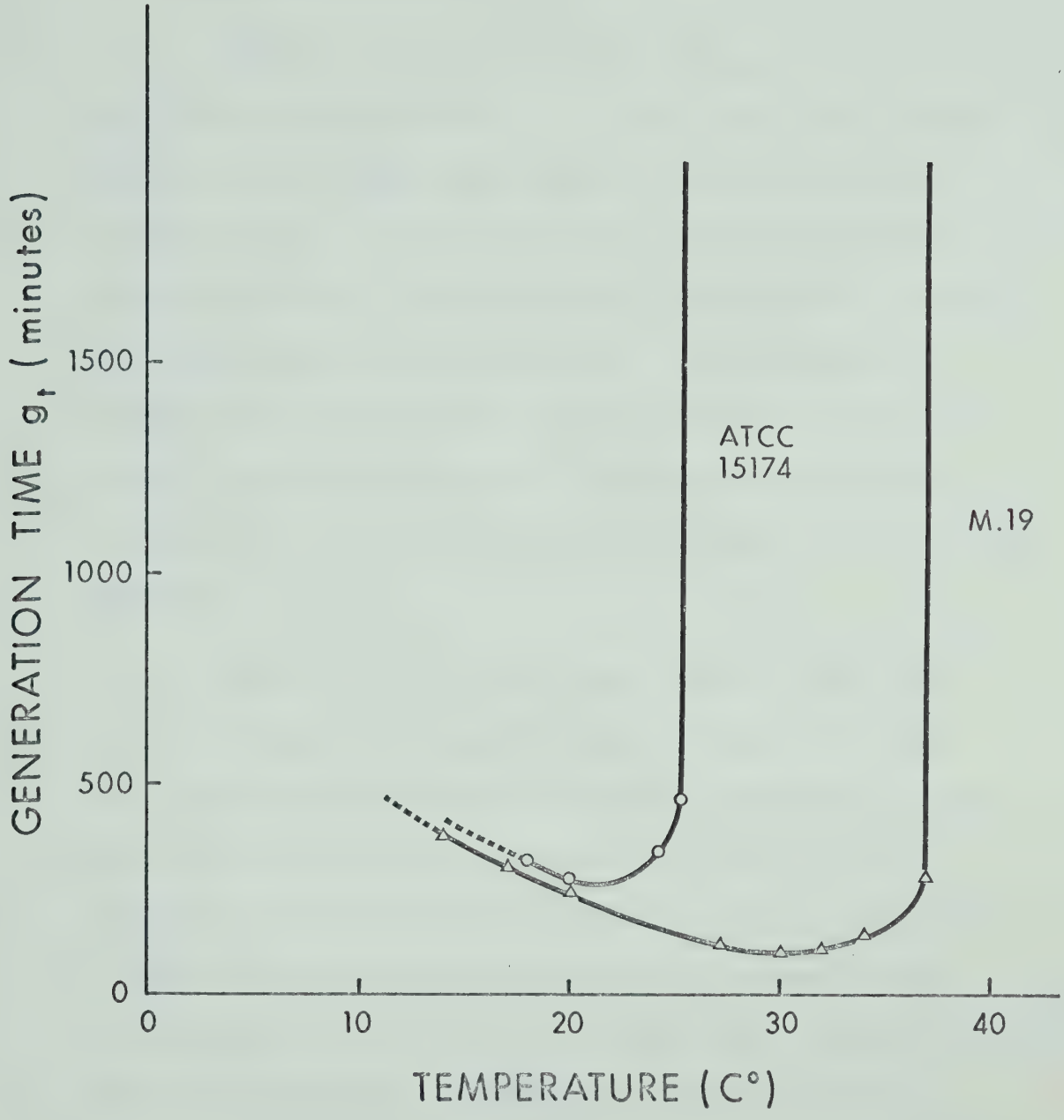


Figure 5.

Effect of Temperature on the Generation Time of M. cryophilus and its Mesophilic Mutant M19.

CELL VOLUME AND CELL COUNTS

Variations in the volume of cells grown at 18, 25 and 27°C are shown in Figure 6. It can be seen that a marked increase in cell volume takes place in the first few hours of growth at all of the temperatures. At 18°C this increase is especially marked and the period of time required to reach the maximum size is somewhat extended. The average cell volumes during the stationary phase at 18°C and 27°C are very similar; the cell volumes at 25°C are however considerably lower during this period, for which no explanation is immediately apparent.

Results for the viable count and total count are shown in Figures 7 and 8 respectively. Although the results are somewhat limited and do not cover all temperatures used in this study they are presented as an aid to later overall evaluation of the data collected in this work. The solid lines in Figure 8 refer to observations on complete growth cycles at the various temperatures. The stippled lines indicate observations subsequent to transfer from 20°C at 48 hours.

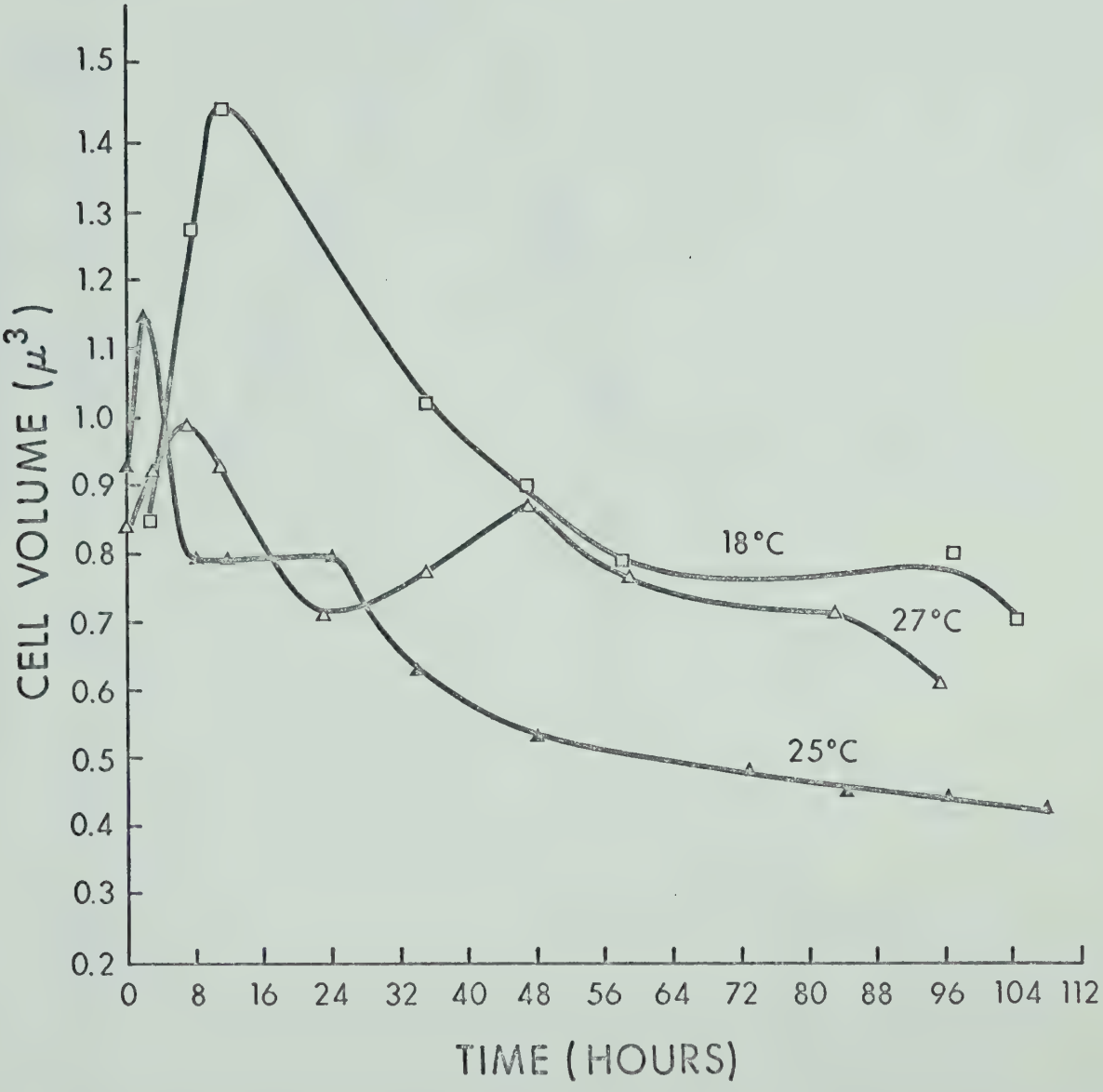


Figure 6.

Effect of Incubation Temperature on Cell Volume of M. cryophilus.

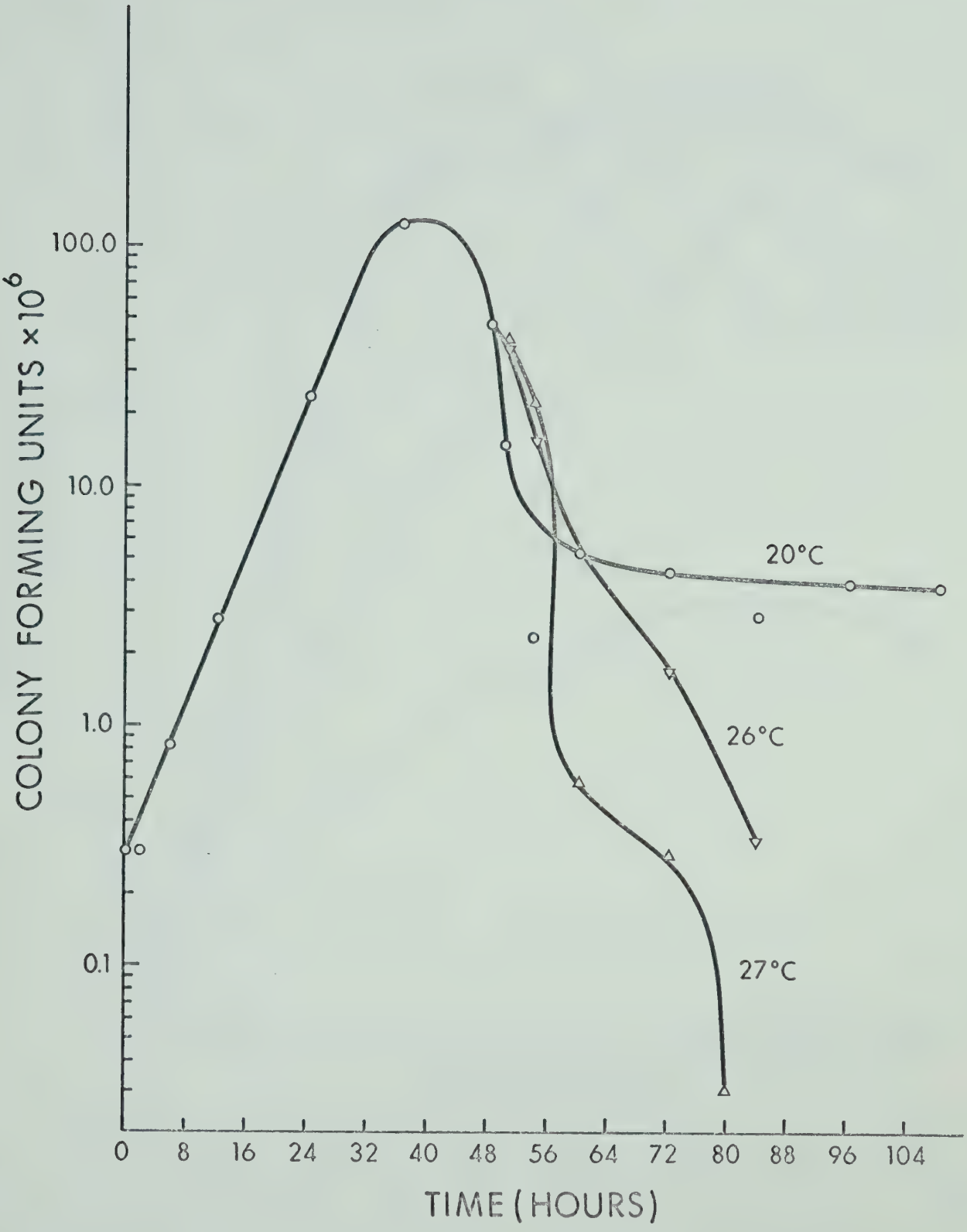


Figure 7.

Effect of a Shift in Incubation Temperature from 20°C to 20, 26 and 27°C on Colony Count of M. cryophilus.

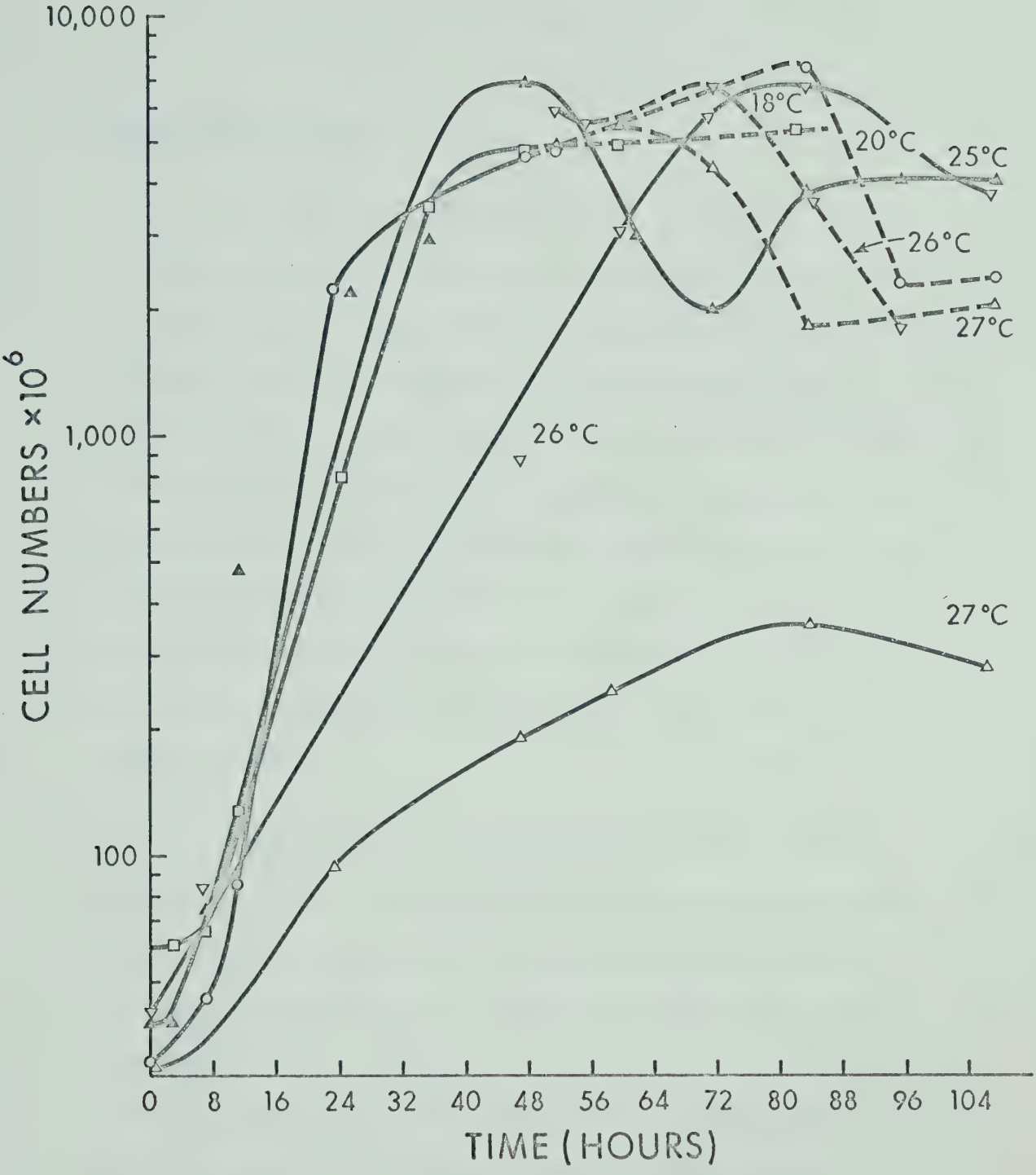


Figure 8.

Effect of Temperature on Cell Count of M. cryophilus.

COMPOSITIONAL STUDIES

The protein and DNA content of the cultures grown at various temperatures were estimated colorimetrically and are illustrated in Figures 9 and 10 respectively; the data represent duplicate determinations on individual samples from at least two complete runs. These curves parallel each other remarkably closely at corresponding temperatures. Only after 24 hours at 28^{o}C was there any marked deviation of the curves from the results obtained for growth in Figure 4. It can be seen from both figures that the content of protein and DNA were maintained at almost maximal level well into the stationary phase.

The RNA content of the cultures grown at various temperatures shows some departure from the pattern observed with protein and DNA Figure 11. Again data are based on duplicate determinations on individual samples from at least two complete runs. The pattern of the curves for RNA is quite similar to protein and DNA to the point at which maximum cell mass is attained, the curves forming the same consistent sequence as before. Beyond this, however, a striking dissimilarity is immediately apparent, the RNA content unlike the previous components is not maintained into and throughout

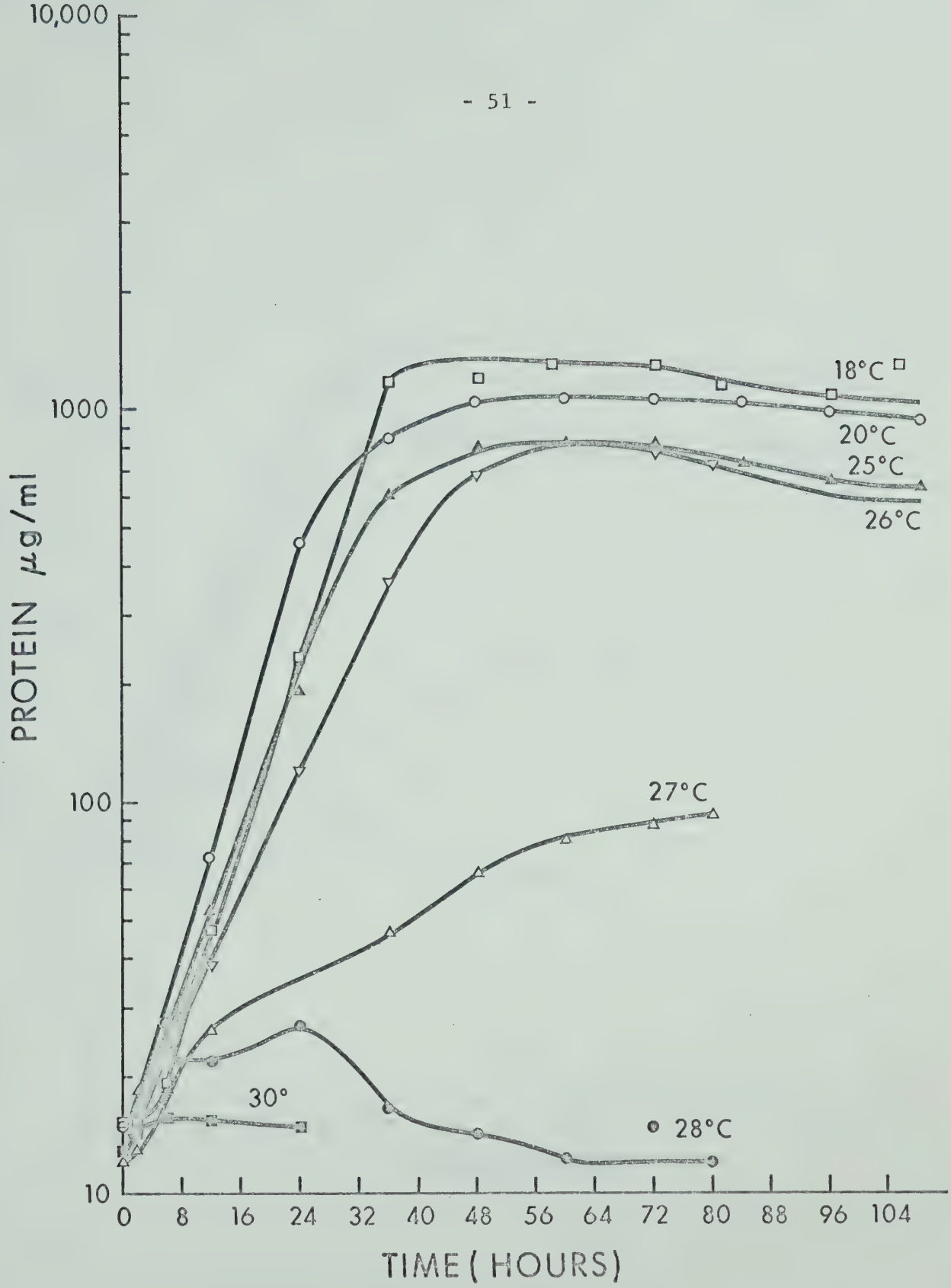


Figure 9.

Effect of Temperature on the Protein Content of Cultures of M. cryophilus.

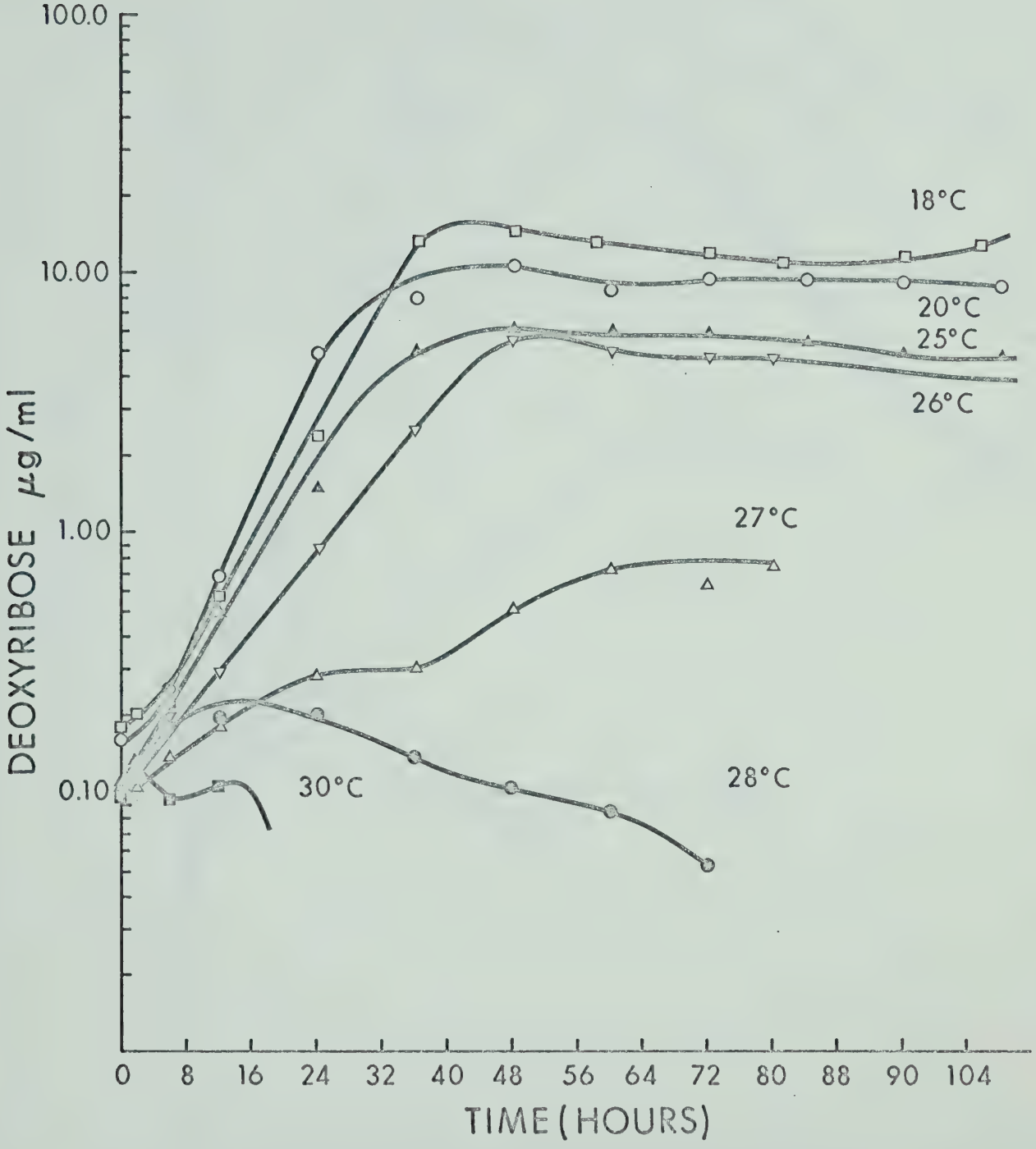


Figure 10.

Effect of Temperature on the DNA Content of Cultures of M. cryophilus.

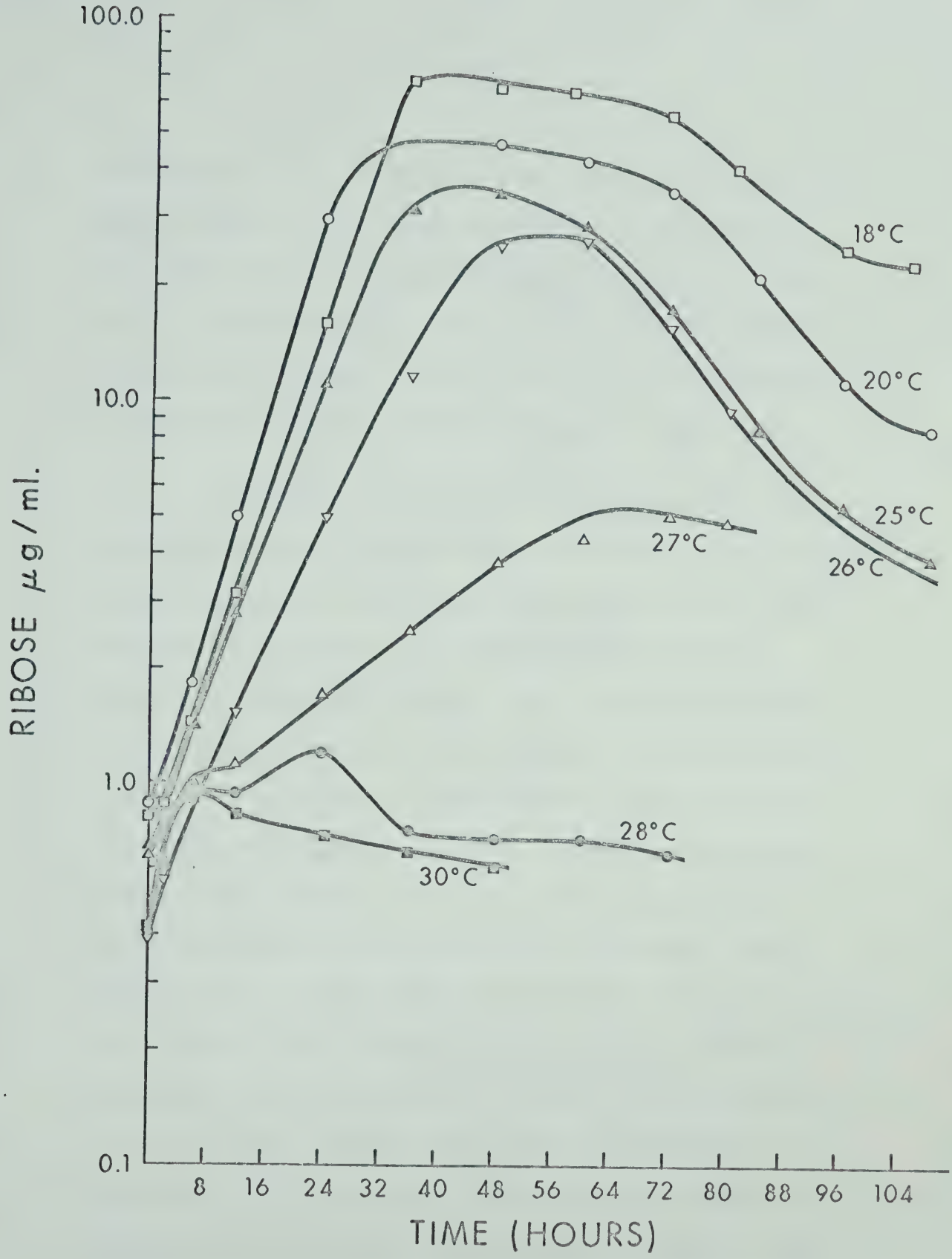


Figure 11.

Effect of Temperature on the RNA Content of Cultures of M. cryophilus.

the stationary phase, but can be seen to fall at progressively greater rates with increasing temperatures. The ribose at 25°C falling from a maximum of 36 µg/ml to 4.4 µg/ml in 60 hours, a reduction of almost 80%. Taking into account the accompanying reduction in cell colume in this time, RNA/cell is reduced to approximately 30% of maximum in this period.

Investigations have shown that over a wide range of growth rates, the fraction of the cell's resources allocated to RNA synthesis is a function of the growth rate at a given temperature, Schaechter, Maaloe and Kjeldgaard, (1958); Neidhardt and Magasanik, (1960). Thus, to study the effect of temperature on macromolecular synthesis and breakdown more closely, it was necessary to distinguish between a growth rate effect and a temperature effect. This was accomplished by growing the cells for 48 hours at 20°C, at which time maximum cell density was attained, and transferring them at this point to the various higher temperatures. The culture thus studied at each temperature was in a similar initial physiological state and possessed the same amounts of protein and nucleic acids. Effects subsequent to transfer were thus attributable to the temperature, and results for such an experiment are presented in Figure 12, 13, 14 and 15; data are based on duplicate determinations on individual samples

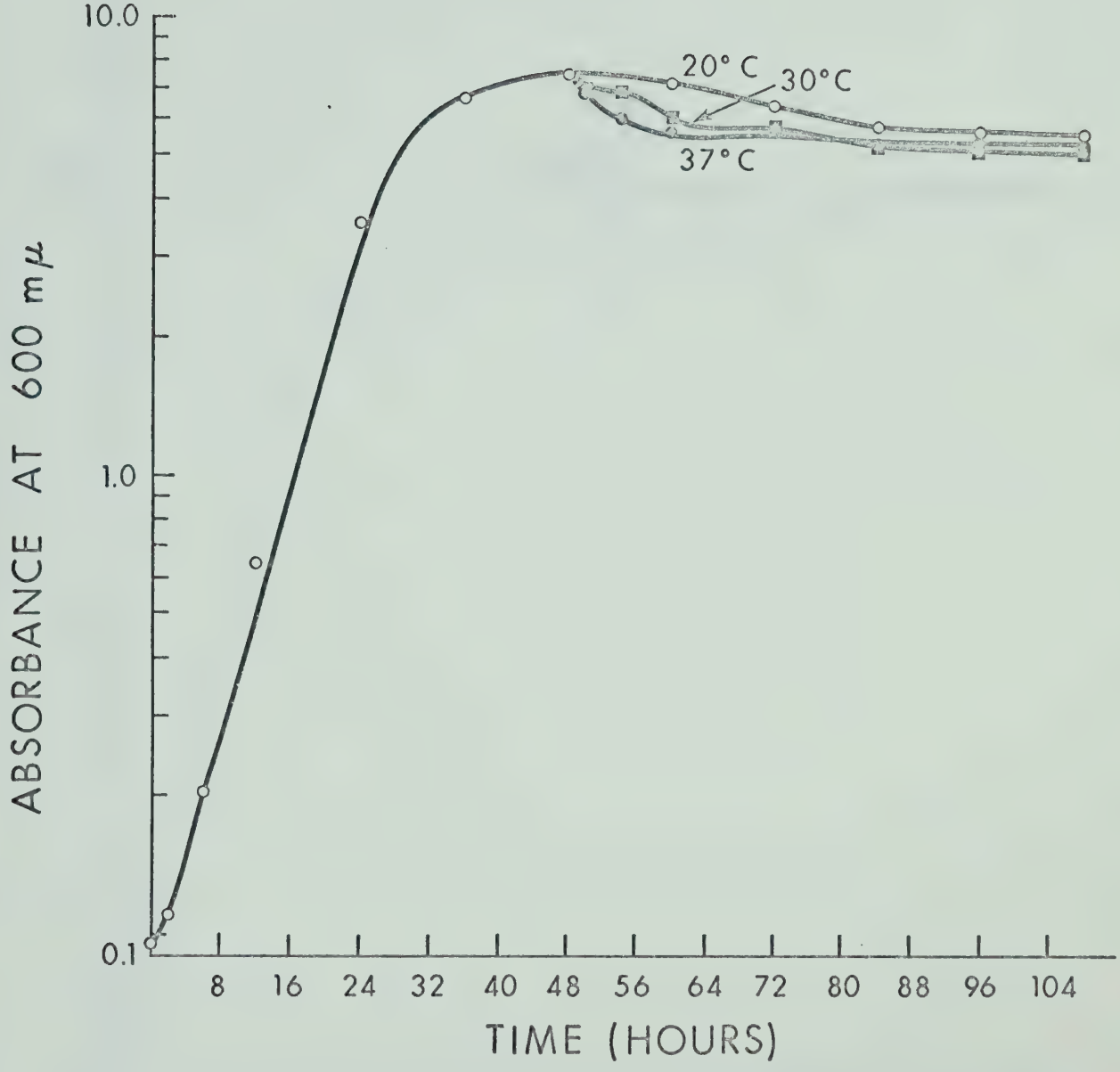


Figure 12.

Effect of a Shift in Incubation Temperature from 20°C to 20, 30 and 37°C on the Growth of M. cryophilus.

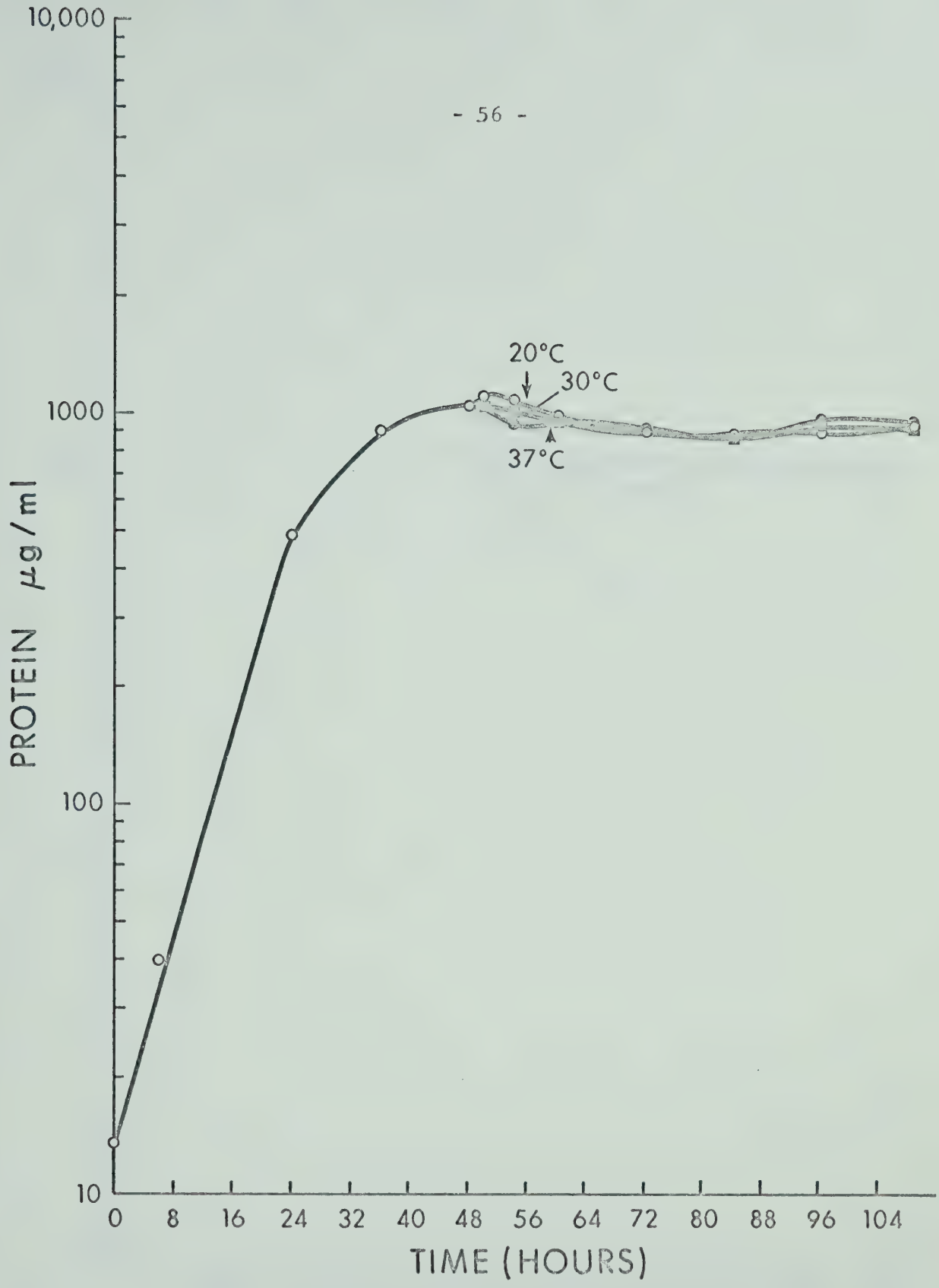


Figure 13.

Effect of a Shift in Incubation Temperature from 20°C to 20, 30 and 37°C on the Protein Content of M. cryophilus.

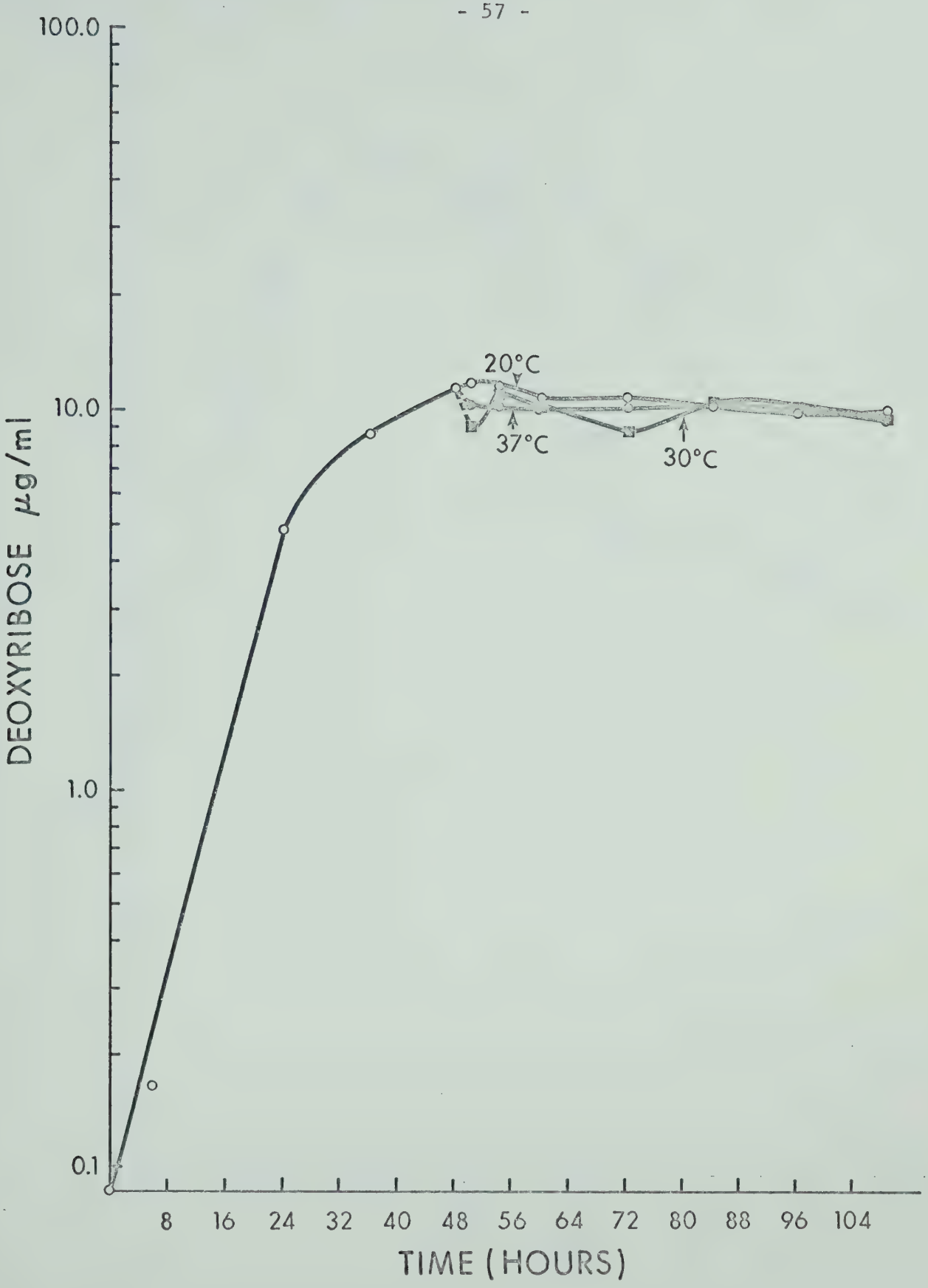


Figure 14.

Effect of a Shift in Incubation Temperature from 20°C to 20, 30 and 37°C on the DNA Content of M. cryophilus.

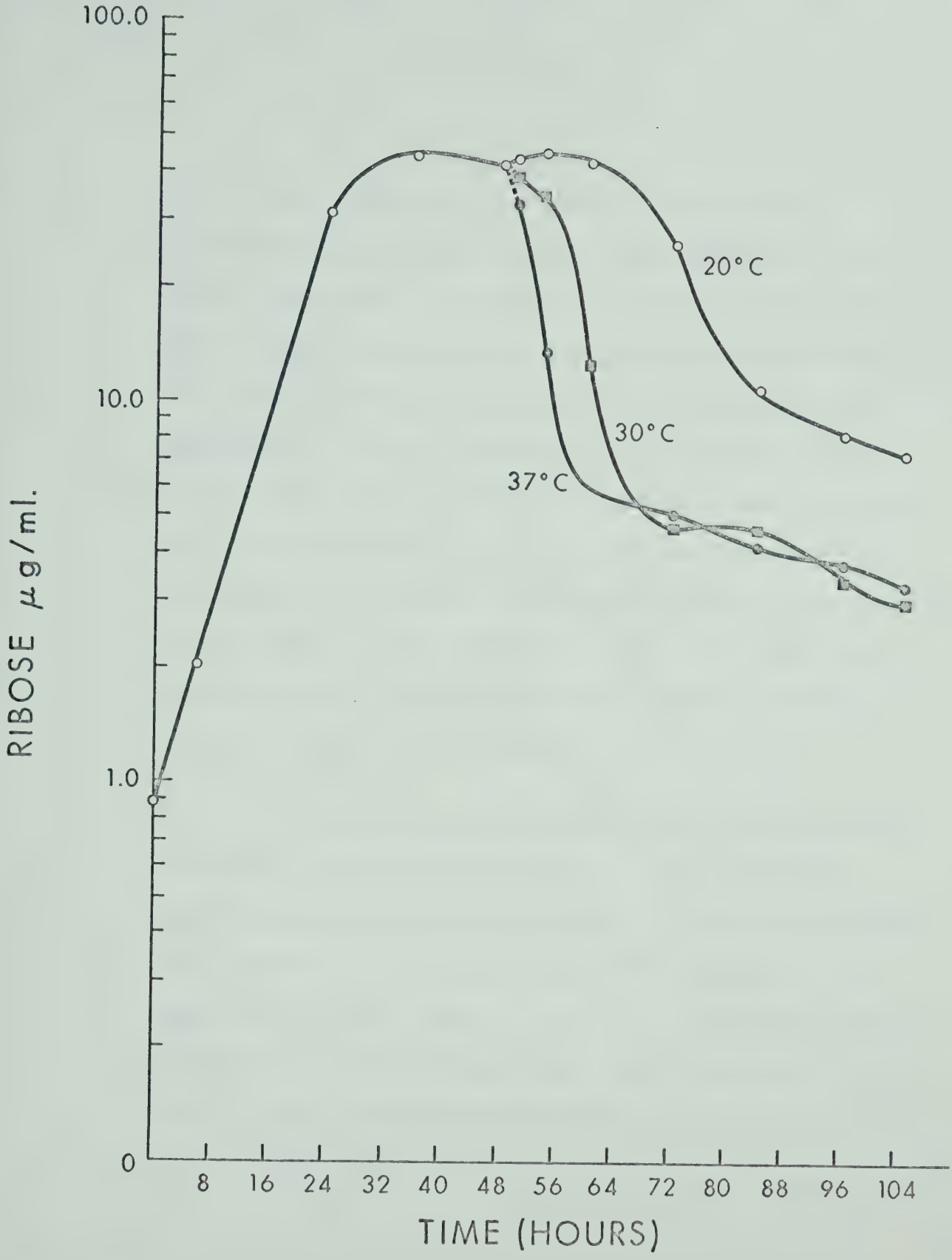


Figure 15.

Effect of a Shift in Incubation Temperature from 20°C to 20, 30 and 37°C on the RNA Content of M. cryophilus.

from various temperatures. Absorbance, protein level and DNA level of cultures remained almost constant at the higher temperatures, and even after an extended period of time the resulting data remained extremely closely alligned. This was not the case with levels of RNA, synthesis and/or degradation of RNA was immediately affected, resulting in a rapid drop in the level of this material. Thus, it appears that this macromolecule is greatly affected by the increased temperature, the relative concentration of RNA and the RNA/100 μg protein Figure 16 both falling. In contrast the DNA/100 μg Protein Figure 17 exhibits much more stability throughout the entire course of the experiment.

A linear plot of the degradation of RNA subsequent to transfer is presented in Figure 18. This demonstrates much more dramatically the actual fall in RNA and the relative rates involved. The optimum rate of RNA degradation would appear to be in the region of 36-37^{o}C as depicted in Figure 19 in the plot of rates of degradation against temperature. This is quite a significant observation the importance of which will be dealt with in the discussion.

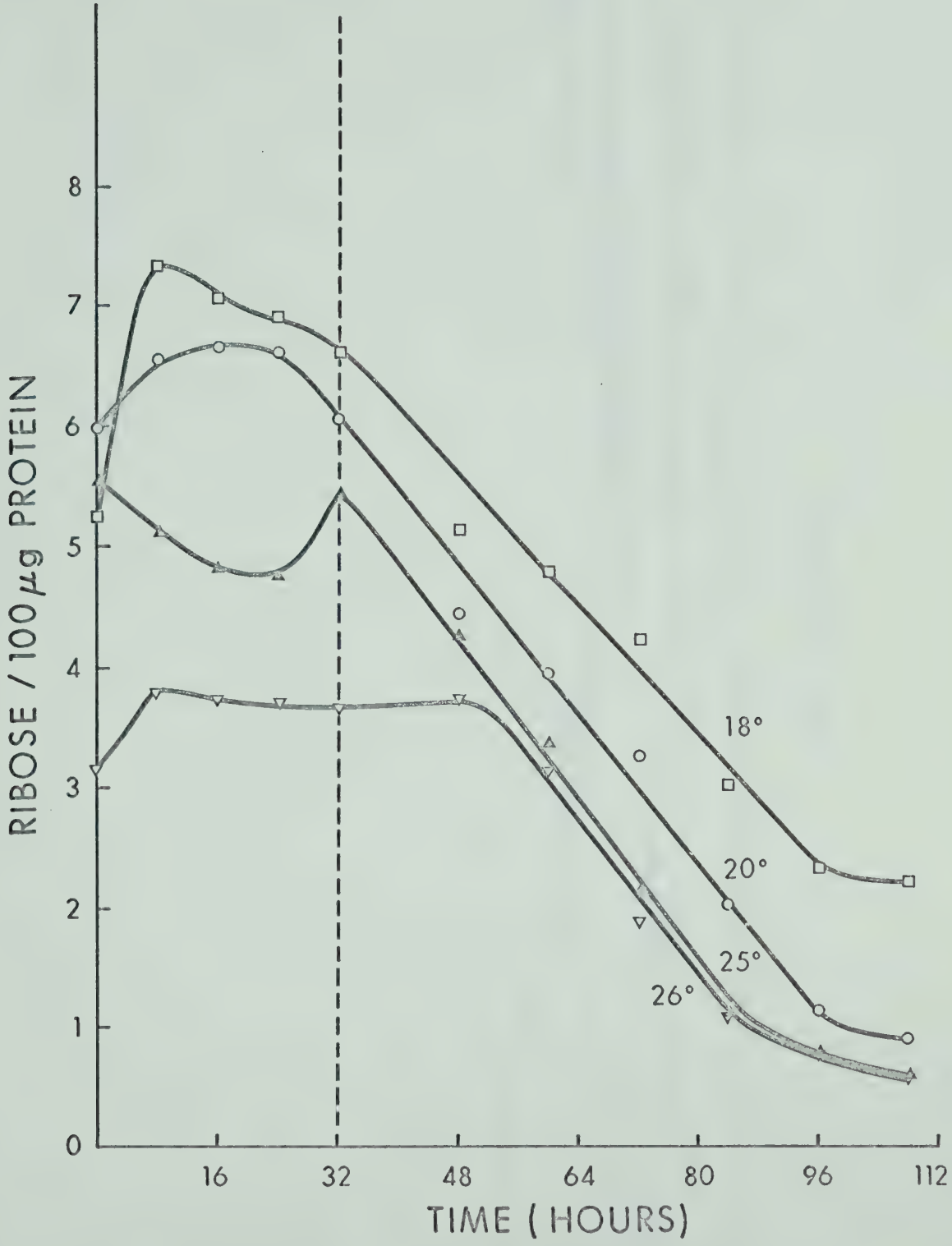


Figure 16.

Relationship between RNA and Protein in Cultures of M. cryophilus as Affected by Temperature of Incubation.

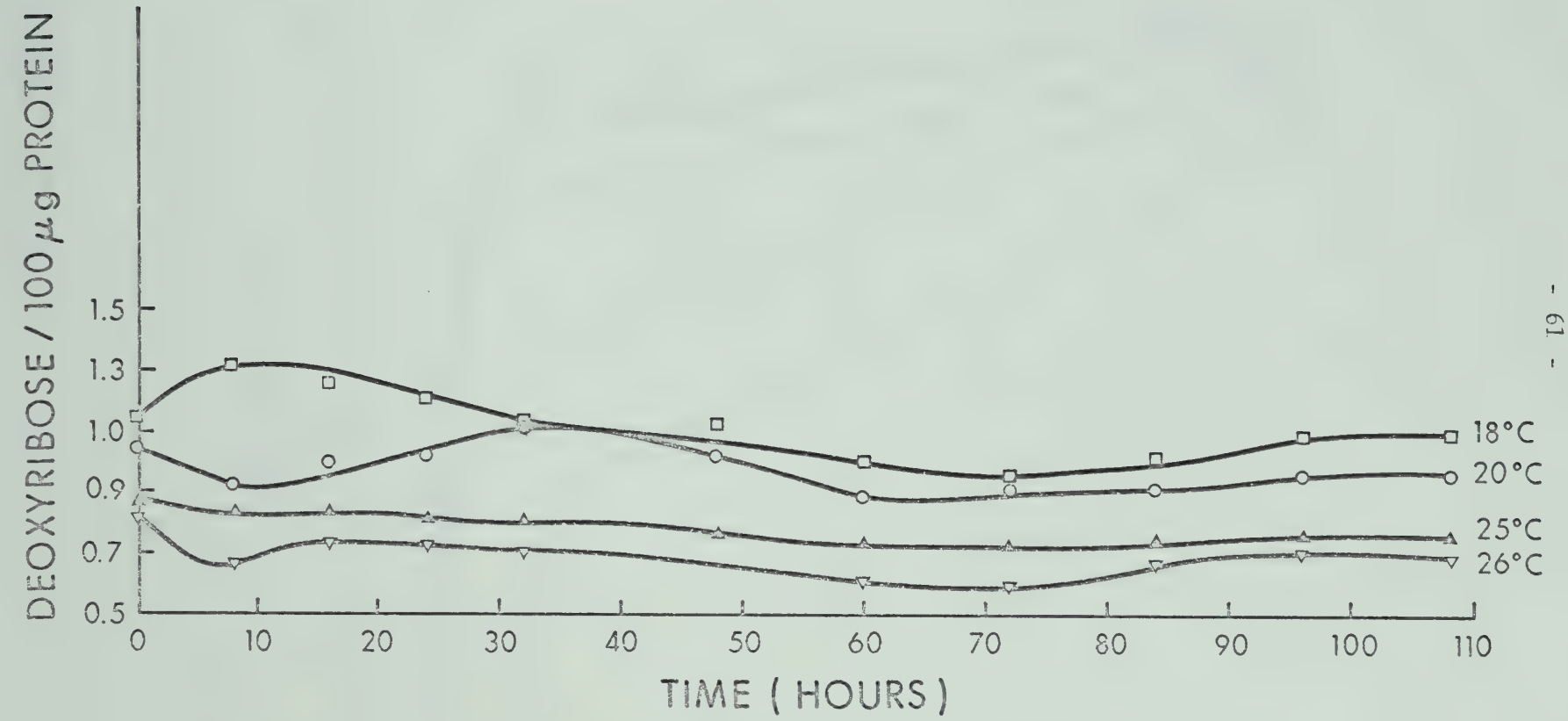


Figure 17.

Relationship between DNA and Protein in Cultures of M. cryophilus as Affected by Temperature of Incubation.

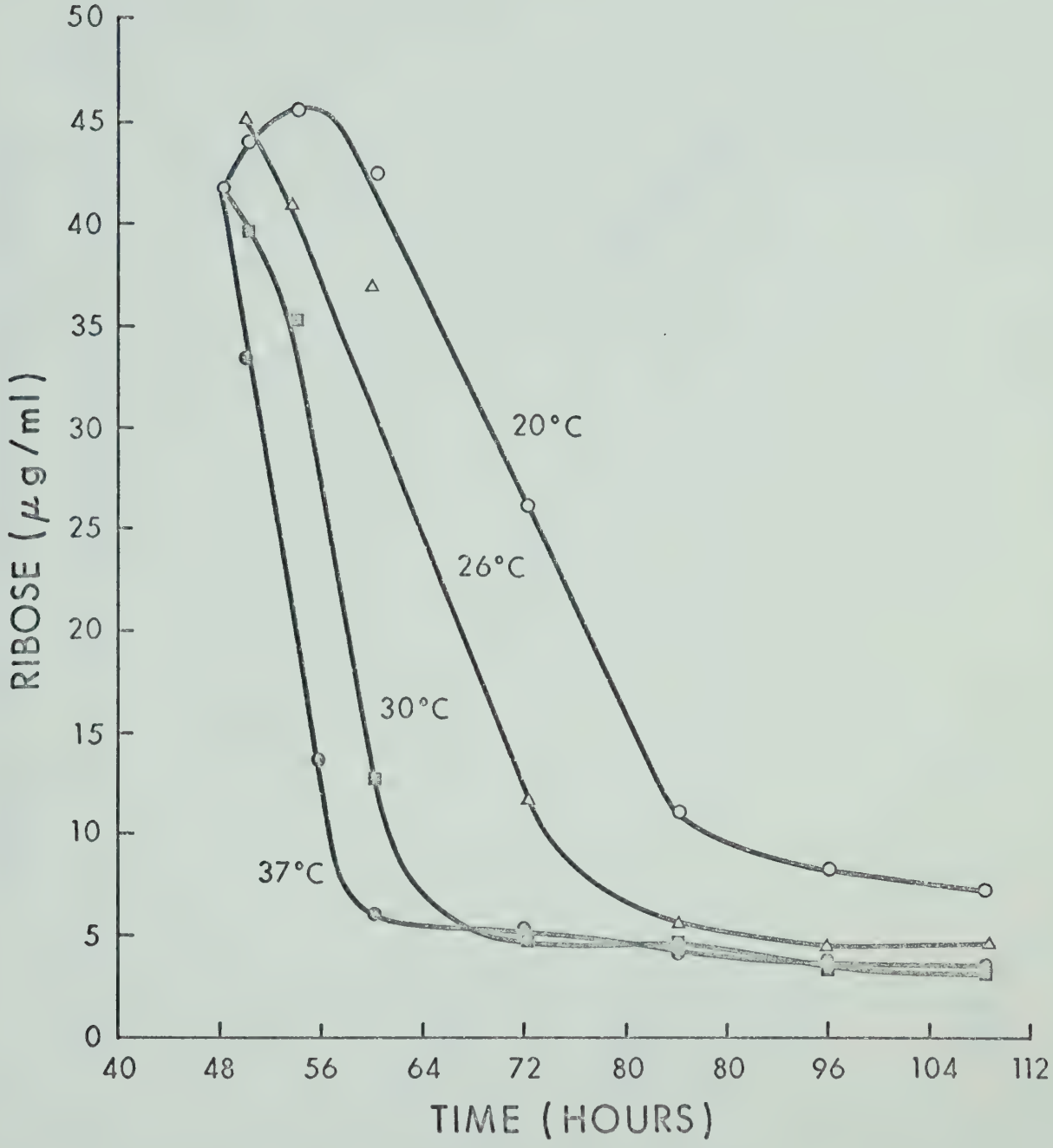


Figure 18.

Effect of Temperature on the RNA Content of cultures of M. cryophilus after transfer from 20°C.

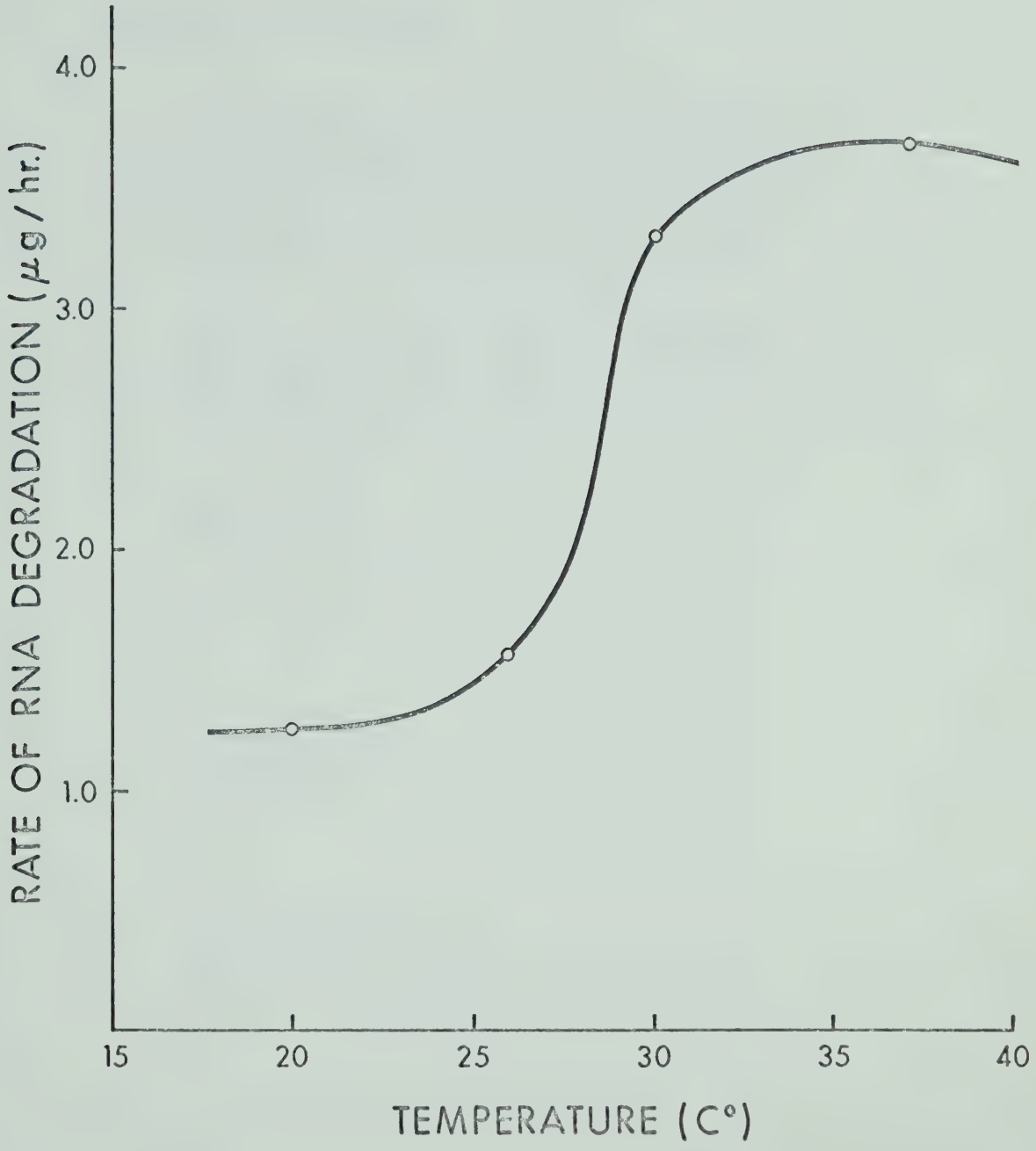


Figure 19.

Effect of Temperature of Incubation on Rate of RNA Degradation in M. cryophilus.

DISC-GEL ELECTROPHORESIS

Electrophoresis of the extracts from logarithmic and stationary cultures at 20, 26 and 27°C produced the pattern shown in Figure 20. A diagrammatic representation of these patterns and the corresponding densitometer traces are provided in Figures 21 and 22 respectively. There is a consistent number of bands revealed in both stages at 20°C and 26°C but reduction in the total number of bands produced was evident at 27°C.

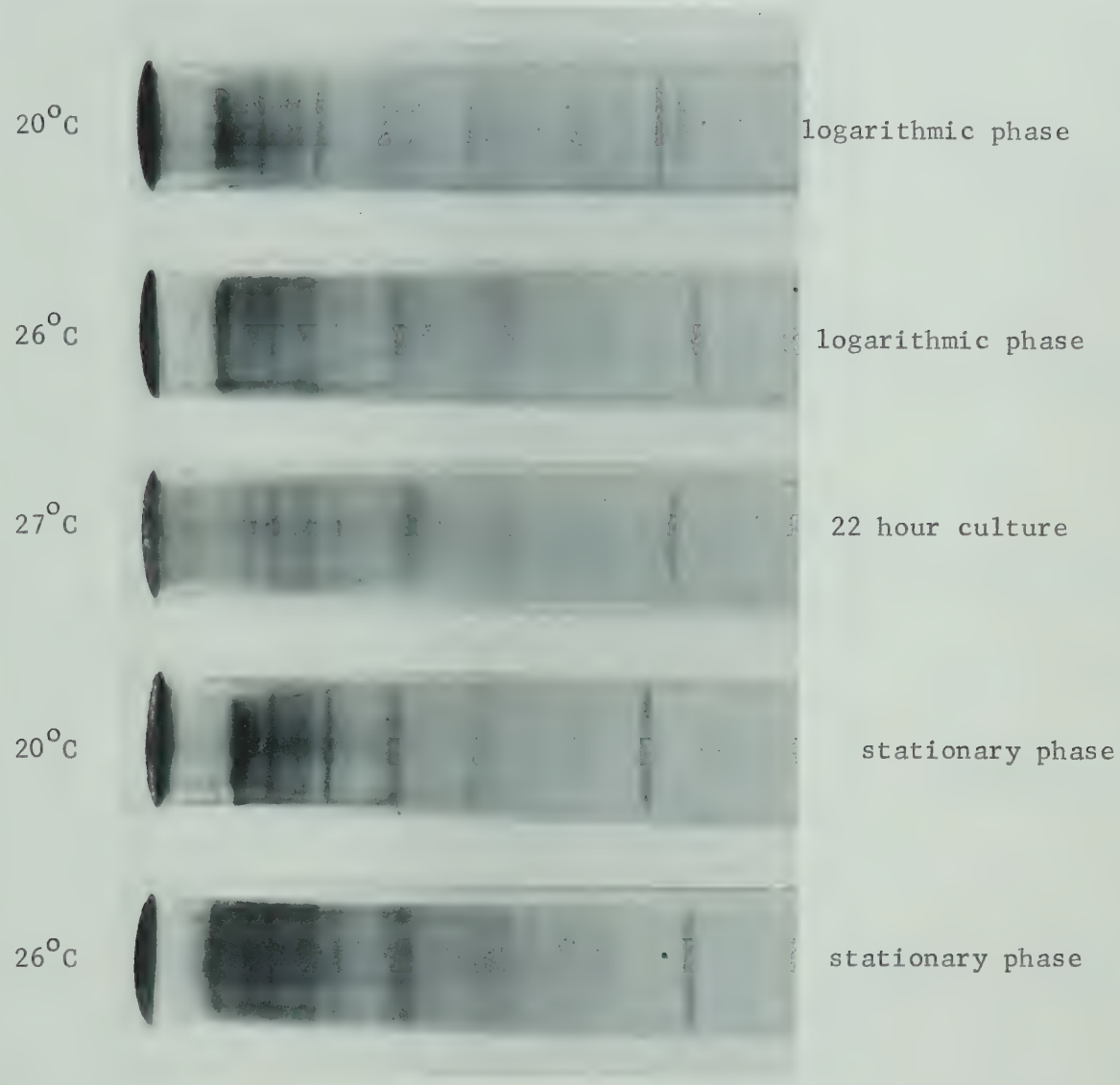


Figure 20.

Effect of Age of Culture and Temperature of Incubation on Souble Protein Pattern of M. cryophilus.

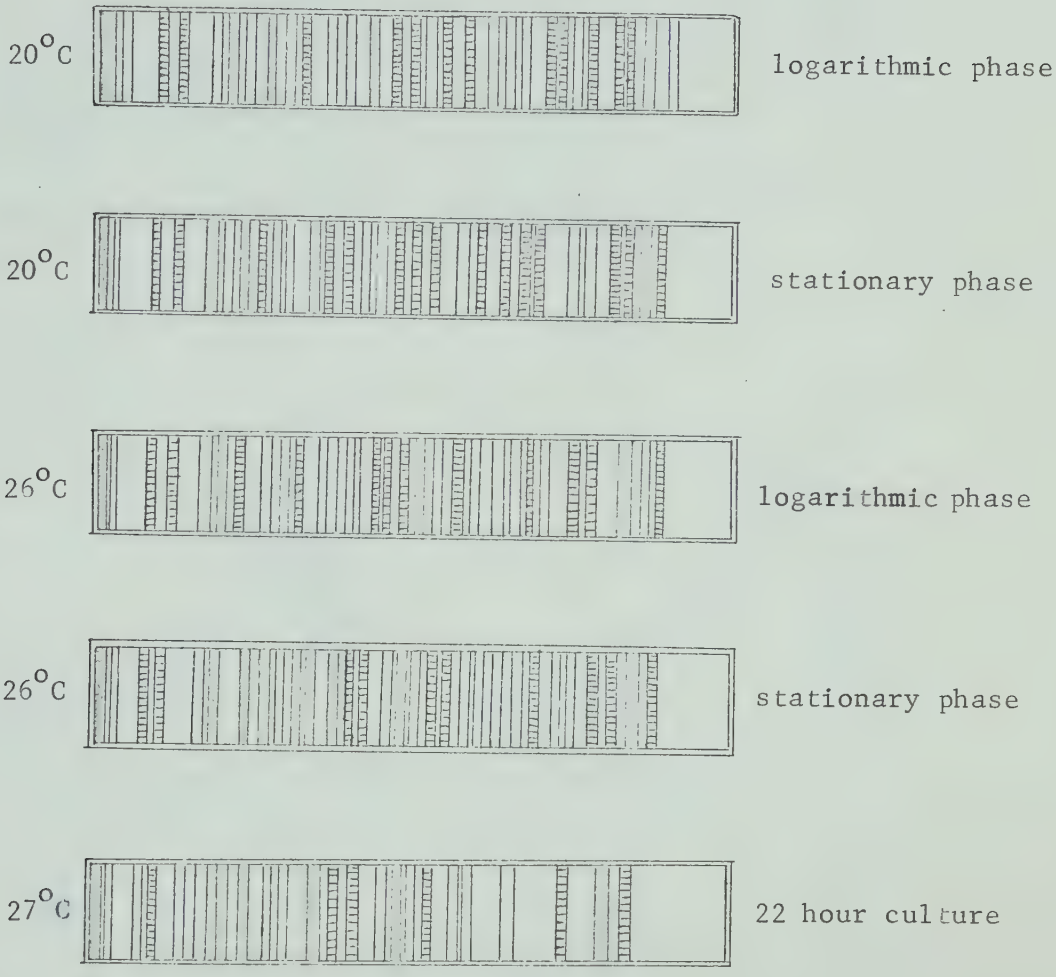
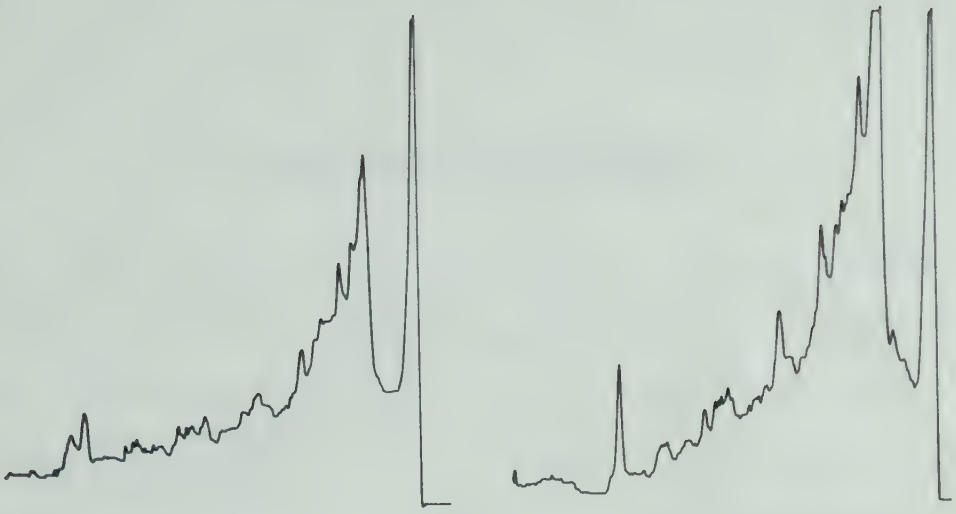
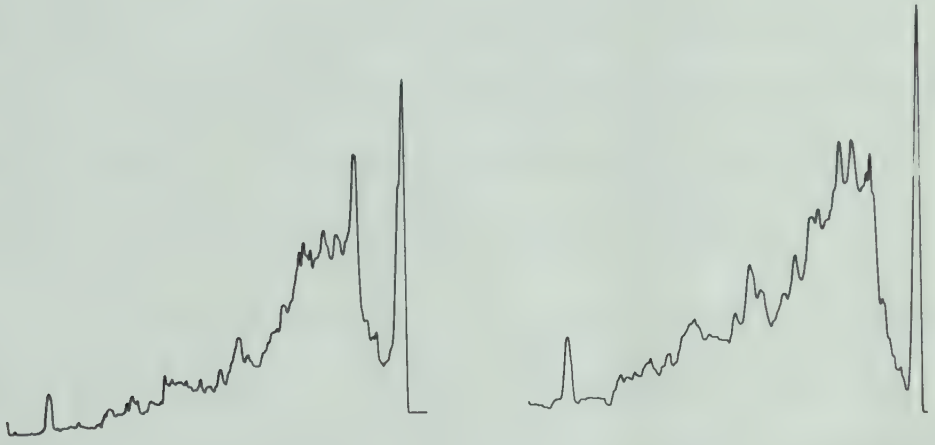


Figure 21.

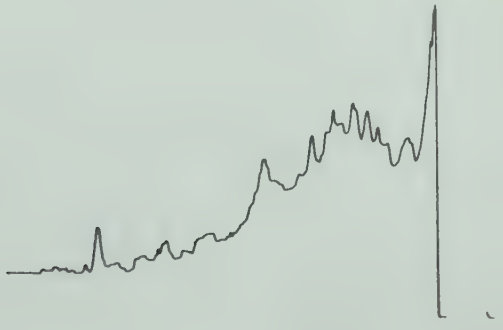
Diagrammatic Representation of Soluble Protein Pattern of M. cryophilus.



logarithmic phase at 20°C stationary phase at 20°C



logarithmic phase at 26°C stationary phase at 26°C



22 hour culture at 27°C

Figure 22.

Densitometric Trace of Soluble Protein Patterns of M. cryophilus

DISCUSSION AND CONCLUSION

This study was primarily concerned with the effects produced by temperature when M. cryophilus is grown within its normal growth range, where the culture exhibits both logarithmic and stationary phases of growth. Further work however, did involve a study of the effects of a shift from 20°C to temperatures in excess of the maximum for growth, on cultures entering the stationary phase. Within the temperatures from 18 to 26°C the cultures exhibit both logarithmic and stationary phases of growth; the effects associated with increases in temperature of such magnitude will now be discussed.

The optimum temperature for growth rate of M. cryophilus is 20°C and as growth rate decreases with either increasing or decreasing temperature, so the rate of macromolecular synthesis decreases. The rates of decrease are not exactly proportional to one another although those at 18 and 20°C are extremely close. As the temperature is increased above 20°C differences in rates become more and more apparent. At an absorbance value of 1 the cell numbers attained at temperatures from 18 to 26°C are relatively constant, as is the protein content per cell. Changes in the cellular content of DNA and RNA were therefore calculated in relation to the protein content. The DNA/100 µg

protein fell by approximately 30% with a temperature increase from 18 to 26°C while RNA/100 µg protein was reduced by approximately 50% Figures 19 and 18 respectively. Both of these decreases while the culture is still in the logarithmic phase of growth are probably due to the reduced growth rates at the higher temperatures, since slower growing cells have a lower DNA and RNA content, Maaloe and Kjeldgaard (1966). The fact that the decrease is not of the same order in each case indicates departure from "balanced growth" defined by Campbell (1957) – "growth is said to be balanced over a time interval if during the interval, every 'extensive' property of the system increases by the same factor. Thus for any one temperature the mass, RNA, DNA and protein demonstrate similar rates of increase."

In the stationary phase the absorbance at 600mµ remains relatively constant and likewise the contents of DNA and protein show no tendency to decrease noticeably. This stability in the level of protein throughout the stationary phase of growth has two possible explanations:

1. There is no protein synthesis at this stage and on the basis of this supposition there must also be no degradation.

2. Net protein synthesis is zero. In other words, the constant protein level is the result of an equilibrium between protein synthesis and degradation.

The relative feasibilities of both explanations will be discussed subsequently.

The DNA level is also extremely stable in the stationary phase and the plot of DNA/100 μg protein for any one temperature exhibits only minor fluctuations throughout the entire growth cycle. From this observed stability and the fact that the denaturation temperature of M. cryophilus is 87.5^{o}C Tai (1967), it is apparent that the determination of maximum growth temperature is not a direct effect on the DNA component of the cell. In relation to this aspect Marmur (1961) reported little connection between maximum growth temperatures and the denaturation temperatures of DNA.

The behaviour of RNA content is very similar to that of the other parameters until the maximum level is attained. At this stage, coincident with the entry of the culture into the stationary phase, the RNA component exhibits a very marked drop, the rate increasing with increasing temperature. During the stationary phase there is only a very gradual reduction in cell volume, thus the observed phenomenal drop in RNA level must be a real fall in cellular content of RNA. This is indicative of greatly intensified RNA degradation induced by the rise in temperature and possibly accentuated by reduction in RNA

synthesis or polymerisation.

Another possibility is RNA utilisation since the fall in RNA content is most marked when the culture has entered the stationary phase. Onset of this phase of growth in a batch culture may occur for several reasons: exhaustion of nutrients, limitation of oxygen, accumulation of toxic metabolic end products. Should growth be limited by the exhaustion of carbon and energy sources the culture may utilise endogenous substrates for maintenance. This phenomenon may thus be responsible for the marked decrease in the level of RNA in the stationary phase cultures. This is supported in part by the data of Tai (1967) that the endogenous respiration of M. cryophilus increases with temperature, though maximum respiration was reported to occur at 25°C.

Tai (1967) showed RNase activity in cell extracts of M. cryophilus to be somewhat constant below 25°C, and to increase rapidly above this temperature to its optimum activity at 37°C, while in contrast to this the RNase activity of a mesophilic mutant M19 was almost constant in this temperature region. These results are in complete agreement with the data obtained for decrease in RNA content in this study, for which the optimum temperature is determined as being 37°C as depicted in Figure 19.

One can see that above 26°C, the maximum growth temperature for this organism, the relative rates increased markedly for a minute temperature rise.

Apparent increase in RNase activity could be the result of temperature exerted conformational changes in the RNA, rendering it more susceptible to attack by the enzyme. Conformational changes have been reported for several types of purified yeast tRNA in the temperature range 22 to 40°C, Sarin et al. (1966). It is thus possible that modification of the active site on the RNA molecule for the nuclease might be involved, or as pointed out by Loftfield and Eigner (1965) the RNA might alter the specificity of the RNase by changing its conformation, as in the case of certain amino acyl-tRNA synthetases. Nevertheless, conformational changes in tRNA are not upheld by the studies of Nash et al. (1969) where 10 tRNA species were tested, none of which exhibited any change in conformation.

The average distribution of RNA components in the cell is approximately 85% ribosomal RNA, 5% tRNA and 3% mRNA. It would appear logical therefore to suppose that this phenomenal decrease in RNA content concerns primarily, if not solely, the ribosomal RNA species. There is extremely rapid turnover

of the mRNA species, for Bacillus subtilus the half life of mRNA at 37°C has been reported as 0.7 minutes Fan, Figer and Leventhal (1964) and it is thus highly unlikely that the mRNA will be affected since it is being replaced so quickly. Transfer RNA is the most stable species and again would not appear to be involved in this fall in RNA content, since reduction of tRNA would result in no protein synthesis taking place. However, from Figure 9, it is evident that protein synthesis must be taking place to maintain the protein content at the constant level shown, in the face of concurrent degradation. Protein synthesis can continue if only the ribosomal RNA species is reduced markedly, since the cell's full complement of ribosomes are not involved in protein synthesis. Thus, a substantial reduction in the number of ribosomes can occur before the rate of protein synthesis is affected and total degradation would be necessary to eliminate protein synthesis completely Strange (1961). The only other possible explanation for the maintenance of the protein level is as mentioned previously i.e. that no synthesis and no degradation of protein is taking place - a rather questionable situation, since the mere fact that the organisms have been growing for 48 hours at this temperature, suggests that there should be no reason to believe, that suddenly protein synthesis is turned off completely by the temperature. The protein level has simply ceased

to increase because maximum cell population has been achieved, but protein synthesis is still able to continue. These findings are similar to those of Schmidt et al., (1956) who observed slow synthesis of protein after RNA synthesis had ceased. These results along with those of the present study, make it clear that cultures can undergo a considerable reduction in RNA content simply by a continued synthesis of other cellular components (e.g., protein and DNA) after DNA accumulation has halted.

Malcolm (1967) presented data on the incorporation of ^{14}C-leucine into cells when transferred from 14 to 30^{o}C. He observed an increase in counts per minute in the cold TCA-insoluble fraction of the cells, which reached a maximum after 15 minutes, then remained stable at this level for the remainder of the test, some 80 minutes. His interpretation of these results was that protein synthesis was active for only 15 minutes after the labelled amino acid was added. Thus he interprets the fact that the counts remain at a constant level for the remainder of the experiment as meaning no protein synthesis is taking place. On the basis of this interpretation, it must follow that no protein degradation takes place at the higher temperature. His data are however open to an alternative interpretation which I should like to submit at this point in the discussion. On addition of the labelled leucine, incorporation

into protein is evident from the initial rise in counts recorded. Since all life processes are the net result of synthesis and degradation, and protein metabolism being no exception, the initial increase in counts is the result of protein being synthesised and protein being degraded. Initially there is no labelled protein to be degraded, thus an apparently high rate of protein synthesis is recorded in the early stages. However, as more and more labelled protein is synthesised greater proportions of the protein being degraded will contain the label, and thus an equilibrium is reached (at 15 minutes), the amount of label incorporation being equivalent to that lost in degradation. The plateau recorded in counts detected, is therefore not necessarily indicative of a halt in protein synthesis, but rather of an equilibrium between protein synthesis and protein degradation. Thus, succinctly, instead of no protein synthesis, as suggested by Malcolm, I submit there is no net protein synthesis.

The importance of this interpretation must not be taken lightly, since if it is agreed that protein synthesis does in fact take place, then obviously the primary site of the temperature induced lesion cannot be inhibition of protein synthesis, as proposed by Malcolm (1967). This also precludes his explanation that the amino acyl-tRNA synthetases are the molecular determinants

of the organisms temperature sensitivity, since again if protein synthesis takes place these activating enzymes must still be functional. Disagreement with this latter explanation is also expounded by Tai (1967), who repeated Malcolm's work using the same method and one of much higher sensitivity. In both instances no evidence was found of inactivation of the amino acyl-tRNA synthetases at 30°C and in fact in most cases the activities at 30°C were higher than at 20°C. Thus in agreement with the present discussion Tai concluded that the heat sensitivity of M. cryophilus was not due to inactivation of the amino acyl-tRNA synthetases, leading to cessation of protein synthesis.

That the RNA component is markedly affected on exposure to elevated temperatures is further confirmed by studies on leakage of material from heat treated cells. Morita and Burton (1963), Haight and Morita (1966) and Kenis and Morita (1968) investigated this aspect using an obligately psychrophilic marine bacterium; Wong et al. (1966) used Arthrobacter citreus for their study; Sogin and Ordal (1967) and Allwood and Russell (1968) studied the effect on Staphylococcus aureus while Langvad and Gokøsyr (1967) were interested in this effect on Meruleus lacrymans, a wood rotting fungi. Iandolo and Ordal (1966) suggested that a series of changes attributable to heat

occurred in Staphylococcus aureus in which weakening of the membrane, coincident loss of soluble pools and simultaneous degradation of RNA were involved. According to Strange and Shon (1964) loss of viability in Aerobacter aerogenes was not due to mere depletion of RNA but instead to increased degradation of RNA and subsequent increase in endogenous pool materials. In all of the studies involving leakage, without exception nucleotides were released from the cells subsequent to the heat treatment. It is thus evident that degradation of polymeric RNA must have taken place. Also the leakage is selective and is thus not associated with a real breakdown of the permeability barriers of the cell.

The electrophoretic study revealed no significant differences in numbers of bands produced from the 20 and 26°C samples, though it was immediately apparent that several bands were absent from the 27°C sample. Superimposition of traces of logarithmic and stationary cultures from corresponding temperatures revealed an interesting point. In stationary cultures the intensity of the bands, with the exception of the first few, was consistently higher than from logarithmic cultures. This difference is no doubt associated with the physiological state of the cultures.

One could expect a change in the enzyme complement of the cells in the region of their maximum growth temperature, and in fact it has been suggested that the difference between mesophiles and their mutants is due to the possession of a different range of soluble proteins. Thus the maximum growth temperature may not be determined by protein synthesis but by proteins synthesised or more precisely by failure to synthesise certain proteins. In support of this, the pattern for 27°C is lacking in several bands, any of which might well be a vital protein whose absence results in the restriction of growth.

The drastic reduction in RNA content with concomitant maintenance of protein, and of DNA to a lesser degree, strongly suggests that the main effect of a temperature increase is directed towards the RNA component of the cell. However, even when the data obtained from this study are supplemented with that available from other reports on this aspect of research, it is still not possible to state precisely the location of the metabolic lesions responsible for the cessation of growth of M. cryophilus above 26°C. The difficulty arises in actually pin-pointing the primary lesion leading to restriction of growth. If we assume for example that death is due to the fact that a certain enzyme is temperature sensitive as suggested by some workers; is the lesion simply at the enzyme level, or is it

directed towards the specific mRNA required for the formation of this enzyme, or does it even occur before this in the form of non-release of the messenger from the DNA? In other words is the lesion a direct result of some phenomenon at the genetic level? These questions or postulates are put forward in an attempt to create a greater awareness of the necessity to consider the complexities involved in biological systems, when attempting to relate a result obtained at the molecular level to phenomena that occur at a cellular level.

It is concluded therefore, that on the basis of the results obtained in this study, the main effect of elevated temperature on M. cryophilus is directed toward the cellular systems associated with RNA. It is probable that the primary effect of the elevated temperature is on the synthesis and/or degradation of RNA.

The fall in the level of RNA that occurs in cultures during the stationary phase of growth would appear to be worthy of further investigation. Although the results of other workers gives strong support to the idea that the RNA is leaking from the cells in the form of nucleotides, other explanations are possible. The RNA may be leaking from the cells as polymeric RNA. Nucleotides may be accumulating in the cell or being utilized endogenously. The actual fate of RNA can only be resolved by further experimentation.

It is apparent that many questions still need to be answered in this very interesting and important area of microbiology.

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